



Review

Liquid biopsies: DNA methylation analyses in circulating cell-free DNA

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ABSTRACT

Analysis of patient's materials like cells or nucleic acids obtained in a minimally invasive or noninvasive manner through the sampling of blood or other body fluids serves as liquid biopsies, which has huge potential for numerous diagnostic applications. Circulating cell-free DNA (cfDNA) is explored as a prognostic or predictive marker of liquid biopsies with the improvements in genomic and molecular methods. DNA methylation is an important epigenetic marker known to affect gene expression. cfDNA methylation detection is a very promising approach as abnormal distribution of DNA methylation is one of the hallmarks of many cancers and methylation changes occur early during carcinogenesis. This review summarizes the various investigational applications of cfDNA methylation and its oxidized derivatives as biomarkers for cancer diagnosis, prenatal diagnosis and organ transplantation monitoring. The review also provides a brief overview of the technologies for cfDNA methylation analysis based on next generation sequencing.

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1. Introduction

In recent years, liquid biopsies have received a great deal of attention owing to their obvious clinical implications. As a noninvasive liquid biopsy, circulating cell-free DNA (cfDNA) is a promising biomarker that provides specific and complementary information in disease diagnosis and prognosis (Wan et al., 2017). Compared to traditional tissue biopsy, cfDNA sequencing has obvious advantages. First, the collection of blood to obtain cfDNA is noninvasive and time unlimited, allowing for real-time and dynamic monitoring of molecular changes for the patients (Xu et al., 2017). Second, cfDNA is released from multiple tumor regions, and may thereby represent the whole molecular picture of a patient's malignancy and solve the problem of intra-tumor heterogeneity (De Mattos-Arruda et al., 2014, 2015; Jamal-Hanjani et al., 2016), which may lead to false-negative results and suboptimal therapy selection (Wan et al., 2017).

DNA methylation is a well-established epigenetic marker that regulates gene expression (Jaenisch and Bird, 2003; Branco et al.,

2011). Altered DNA methylation patterns are early events in many tumors (Irizarry et al., 2009; Baylin and Jones, 2011, 2016). cfDNA includes both genetic and epigenetic information. It is reported that circulating tumor DNA (ctDNA) bears cancer-specific methylation patterns (Board et al., 2008). These observations reveal that cfDNA methylation is a new promising biomarker.

1.1. Aberrant DNA methylation in cancer

DNA methylation is a kind of epigenetic modifications, which adds a methyl group to the 5th carbon of cytosine (5-methylcytosine, 5mC) by DNA methyltransferases (DNMTs) and prefers to occur within CpG dinucleotides (Bird, 2002; Jones, 2012). 5mC can be oxidized to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) by ten-eleven translocation (TET) proteins. The latter two are excised by thymine DNA glycosylase (TDG) coupled with base excision repair (BER), which results in unmodified cytosine (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009; Ito et al., 2010; He et al., 2011; Maiti and Drohat, 2011; Pfaffeneder et al., 2011). This process is known as active DNA demethylation (Fig. 1). The dynamic regulation of DNA methylation is a very vital process in cell fate determination and development (Feng et al., 2010). In somatic cells, 5mC is primarily restricted to palindromic CpG dinucleotides, and

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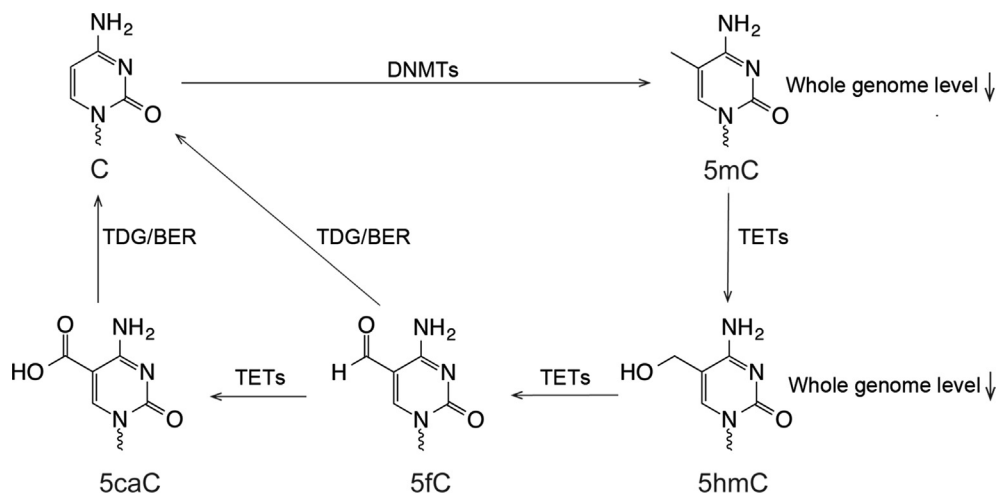


Fig. 1. The dynamic regulation of DNA methylation and aberrant DNA methylation in cancer. DNA methyltransferases (DNMTs) convert unmodified cytosine to 5-methylcytosine (5mC). Ten-eleven translocation (TET) enzymes can oxidize 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). Thymine DNA glycosylase (TDG)/base excision repair (BER) pathway excises 5fC/5caC and regenerates unmodified cytosine. In cancer cells, the whole genome levels of 5mC and 5hmC are decreased.

about 60%–80% of CpGs are methylated (Smith and Meissner, 2013). Generally, methylated DNA is mainly enriched at promoters and is correlated with inhibition of transcription initiation, so DNA methylation has been known as a repressive marker in genome (Bird, 2002).

Aberrant DNA methylation is a noticeable feature of cancer cells (Esteller, 2007; Baylin and Jones, 2011). The cancer genome is globally hypomethylated (Fig. 1), while hypermethylation of tumor suppressor genes is an early event in many tumors, which promotes cancer progression (Bird, 2002; Baylin and Jones, 2011). Growing evidence suggests that impairment of active DNA demethylation may contribute to cancer initiation (Cimmino et al., 2011; Wu et al., 2011). Active DNA demethylation is TET-mediated. TET1 was initially identified owing to its fusion to mixed-lineage leukemia (MLL) in patients with acute myeloid leukemia (AML) (Ono et al., 2002). Around the time when TET-mediated oxidation of 5mC was discovered, multiple studies reported TET2-inactivating mutations in myeloid disorders (Delhommeau et al., 2009; Tefferi et al., 2009). In addition to TET2 mutations, TET1 and TET3 mutations were observed in haematopoietic malignancies (Abdel-Wahab et al., 2009). Mutations of TET proteins are also observed in various solid tumors, which causes aberrant active DNA demethylation, as shown by the reduction of 5hmC (Lian et al., 2012; Yang et al., 2013; Thienpont et al., 2016; Niu et al., 2017). Therefore, global decrease of 5hmC may be broadly used as a diagnostic biomarker for cancers (Hsu et al., 2012; Lian et al., 2012; Yang et al., 2013). As to another enzyme in demethylation, TDG has also been implicated in various cancers (Dalton and Bellacosa, 2012), although it may be due to TDG's role in mismatch repair.

1.2. cfDNA biology

The presence of fragments of cell-free nucleic acids in human blood was initially reported in 1948 (Mandel and Metais, 1948). In addition to the blood circulating system, cfDNA has also been detected in various body fluids, including urine (Botezatu, 2000; Chan et al., 2008), saliva (Mithani, 2007), pleural fluid (Sriram et al., 2012) and cerebrospinal fluid (De Mattos-Arruda, 2015; Pan et al., 2015; Wang, 2015). cfDNA is a mixture of extracellular nucleic

acid fragments originating from cell necrosis, apoptosis, and active release of DNA (Stroun et al., 2000). The modal length of cfDNA has been determined as around 160–180 bp (Jahr, 2001; Lo, 2010; Thierry, 2010), corresponding with the unit size of nucleosome. In healthy individuals, the concentration of cfDNA in plasma ranges from 1 to 10 ng/mL of plasma (Mouliere et al., 2011, 2014). The level of cfDNA may be increased in the state of infection (De Vlamincq, 2015), tissue damage (Rodrigues Filho, 2014) or cancer (Leon et al., 1977). Recent studies suggested that cfDNA has potential biological functions such as stimulation of cell transformation and tumorigenesis of recipient cells (Garcia-Olmo et al., 2010; Trejo-Becerril et al., 2012), and induction of neutrophil release into blood to clear infected bacteria (Kaplan and Radic, 2012; Gould et al., 2015).

2. Diagnostic application of cfDNA methylation

In 1989, Stroun and colleagues reported that cfDNA in the plasma of cancerous patients contains tumor DNA originating from cancer cells (Stroun et al., 1989). After that, a large amounts of genetic and epigenetic alterations in cfDNA have been reported (Schwarzenbach et al., 2011). Especially, in recent years, liquid biopsies have gained increasing attention, as they are convenient and minimally invasive means to gain fetal or tumor DNA for noninvasive prenatal testing, tumor screening and monitoring of treatment response. cfDNA-bearing specific methylation patterns, which are consistent with the cells where they originate, have been investigated as feasible biomarkers (Board et al., 2008).

2.1. ctDNA methylation as biomarkers for cancer

Circulating tumor DNA (ctDNA), which originates from cancer cells, represents a substantial fraction of cfDNA, which ranges from <0.05% to 90% depending on tumor localization, size and vascularization, and clearance of liver and kidneys (Jahr, 2001; Fleischacker and Schmidt, 2007; Diehl et al., 2008). The half-life of ctDNA in circulation is between 16 min and 2.5 h (Lo, 1999; To, 2003; Diehl et al., 2008; Wang et al., 2016), thus allowing for real-time and dynamic monitoring of tumor statuses. Recent

studies have determined that the methylation patterns of ctDNA are different from those of normal cfDNA and blood leukocytes (Guo et al., 2017; Xu et al., 2017). Abnormal DNA methylation alterations in ctDNA are associated with tumorigenesis (Gold et al., 2015), so they can be biomarkers for cancer diagnosis. ctDNA methylation analysis has advantages over somatic mutation analysis for cancer diagnosis, such as higher clinical sensitivity and dynamic range. In addition, methylation markers are conserved in cancer tissue and ctDNA, whereas some mutations in cancer tissue may not be detected in ctDNA (Xu et al., 2017).

Many ctDNA methylation biomarkers for cancer have been reported, and they have recently been reviewed elsewhere (Jung et al., 2010; Schwarzenbach et al., 2011; Lissa and Robles, 2016). These methylation markers mainly depend on the methylation level of individual CpG sites, and their sensitivity is limited by the technical difficulties in measuring single-CpG methylation. To increase the signal-to-noise ratio of ctDNA methylation analysis, the regional nature of DNA methylation has been exploited (Lehmann-Werman et al., 2016). Based on the assumption that adjacent CpG sites have the same methylation situation, two recent reports identified cancer-specific methylation marker panels and their results demonstrated the utility of ctDNA methylation markers in cancer diagnosis (Guo et al., 2017; Xu et al., 2017). Another DNA modification 5hmC has also been reported to be a cancer biomarker, and has achieved about 80% detection sensitivity which was much higher than that of classical biomarkers and epidemiological factors (less than 50%).

2.2. cffDNA methylation as biomarkers for prenatal diagnosis

The linkage between advanced maternal age and Down syndrome (trisomy 21), Edward syndrome (trisomy 18) and Patau syndrome (trisomy 13) has been reported (Allen et al., 2009). Traditional aneuploidy screening method is amniocentesis, which has highly accuracy. However, amniocentesis would cause trauma and carries a risk of miscarriage. In 1997, Lo (1997) reported the presence of cell-free fetal DNA (cffDNA) in maternal plasma arising from nutrient exchange through placenta. cffDNA displays a predominant peak of around 143 bp (Lo et al., 2010), and comprises 10%–15% of the total plasma cfDNA (Wang et al., 2013). cffDNA can be detected by basic molecular techniques in maternal plasma as early as five weeks of gestation (Guibert et al., 2003), and its level depends on multiple pregnancy, gestational weight and maternal age (Kinnings et al., 2015).

The principle of noninvasive prenatal testing (NIPT) for trisomy 21 is the detection of an additional number of DNA fragments derived from fetal chromosome 21 in the maternal plasma. cffDNA can be distinguished from maternal background DNA for its unique aspects such as fetal-specific SNPs and epigenetic markers (Poon et al., 2002; Zimmermann et al., 2012). Fetal-specific epigenetic markers such as DNA methylation, which differ from maternal DNA background, can be used to estimate the number of fetal chromosome 21 (Lui et al., 2003; Ng et al., 2003). In 2005, the first universal DNA methylation marker of cffDNA was reported (Chim et al., 2005). In this study, the *maspin* gene presented different DNA methylation distributions between placenta and maternal blood cells. Then other DNA methylation markers of cffDNA in maternal plasma were found and could help to detect trisomy 21 and trisomy 18 (Tong et al., 2006; Old et al., 2007; Chim et al., 2008). More and more novel universal fetal-specific methylated CpG sites, which may be used in clinical practice, were identified through whole genome bisulfite sequencing (WGBS) strategy (Lun et al., 2013). These methylation biomarkers provide more information about the fetus, which can help to monitor the condition of the placenta and improve the detection rate.

2.3. cfDNA methylation as biomarkers for organ transplantation monitoring

Organ transplantation is a widely used medical procedure, while it carries risks of infection and rejection. Thus, it is essential to monitor the condition of allograft to make sure that transplantation is effective. Following organ transplantation, the plasma of recipients presented donor-derived cfDNA (dd-cfDNA) from transplanted liver and kidney (Lo et al., 1998). Later studies illustrated that dd-cfDNA could serve as biomarkers of transplant injury (Gadi et al., 2006; Moreira et al., 2009; Schtz et al., 2017). The ratio of dd-cfDNA to the total cfDNA in plasma can estimate the recovery status and can be used to guide the therapeutic decisions (Daly, 2015; Burnham et al., 2017). Through tracing the origin of cfDNA, clinician can accurately monitor the recovery of donor organ. A method named “genome transplant dynamics (GTD)”, which depends on SNP distribution, has been developed to distinguish donor and recipient cfDNA molecules in plasma (Snyder et al., 2011). On the other hand, as DNA methylation is heterogeneous between different people, cfDNA methylation markers can also provide information for distinction between donor and recipient cfDNA (Sun et al., 2015). Thus, cfDNA methylation detection can be used for organ transplantation monitoring, which bears the advantage of being noninvasive compared with the classic diagnosis for rejection. Take heart transplant recipients as an example, the gold standard for diagnosis of their rejection is the endomyocardial biopsy which is invasive and may cause arterial puncture and biopsy-induced tricuspid regurgitation.

3. cfDNA methylation analysis methods

Bisulfite sequencing is regarded as the gold standard for 5 mC detection, which transforms non-methylated cytosines to uracils after sodium bisulfite treatment while does not alter methylated cytosines (Frommer et al., 1992). Following DNA sequencing, DNA methylation sites can be detected. Sodium bisulfite treatment would cause a degree of DNA degradation which may lead to loss of some critical information (Tanaka and Okamoto, 2007). In order to apply bisulfite sequencing to highly fragmented cfDNA, library preparation and treatment process have been optimized. In this review, we summarize the cfDNA methylation detection methods based on next generation sequencing (Fig. 2).

3.1. Whole genome bisulfite sequencing (WGBS)

WGBS can provide single-base resolution information without biases, which was developed to map the epigenome in *Arabidopsis* and detect human DNA methylomes (Lister et al., 2008, 2009). In this approach, the addition of adaptor sequences to both ends of cfDNA fragments before bisulfite treatment guarantees that preligated cfDNA can be amplified (Fig. 2). WGBS has been instrumental for mapping cfDNA methylation in plasma (Chan et al., 2013; Lun et al., 2013; Legendre et al., 2015). This approach provides genome-wide information, while the required depth of sequencing is generally high and the cost of sequencing is very expensive.

3.2. Single-cell reduced-representation bisulfite sequencing (scRRBS)

DNA methylation occurs predominantly in CpG dinucleotides, the occurrence of which in vertebrate genomes is lower than that of other dinucleotides. Genomic regions rich in CpG dinucleotides called CpG islands (CGIs). There is a correlation between gene silencing and the methylation of CGIs. Selection for biologically

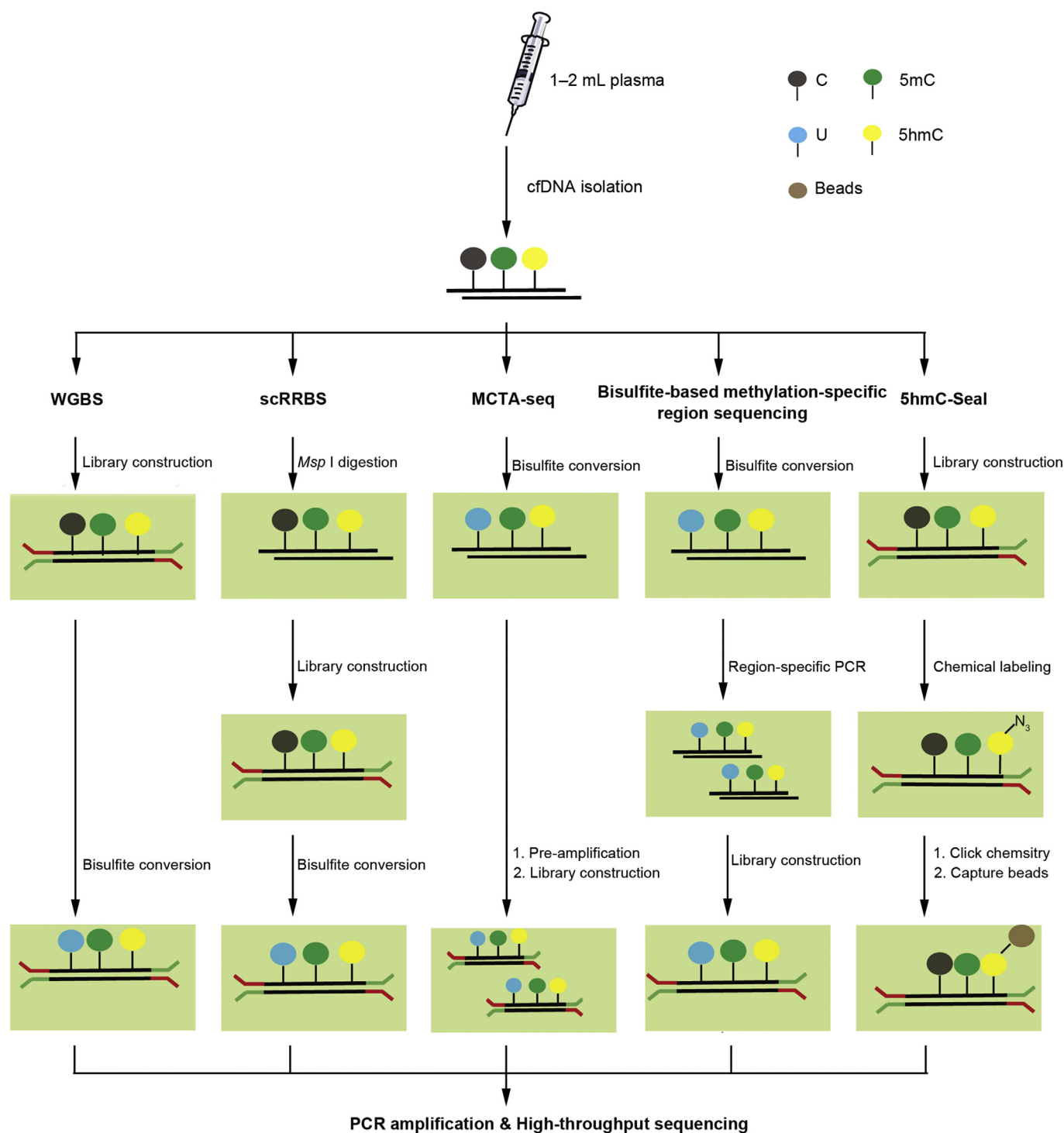


Fig. 2. Schematic diagram of procedures of different cfDNA methylation analysis methods. cfDNA is isolated from plasma and then subjected to different DNA methylation analysis methods, including whole genome bisulfite sequencing (WGBS), single-cell reduced-representation bisulfite sequencing (scRRBS), methylated CpG tandems amplification and sequencing (MCTA-seq), bisulfite-based methylation-specific region sequencing and 5hmC-Seal.

relevant CGIs before sequencing can reduce the sequencing requirements. Reduced-representation bisulfite sequencing (RRBS) is an approach being developed based on this strategy, which makes use of a methylation-insensitive restriction enzyme *Msp* I to select for CpG-rich sequences (Meissner et al., 2008). Single-cell RRBS (scRRBS) is an adaptation to RRBS which reduces the required input

to ~1 ng of DNA (Guo et al., 2013). Recently, scRRBS has been applied to detect cfDNA methylation (Guo et al., 2017). This approach performs adapter ligation immediately after DNA fragmentation by restriction enzyme *Msp* I, and then performs the bisulfite conversion of the ligated DNA. After this procedure, the purified DNA is amplified and subjected to deep sequencing (Fig. 2).

3.3. Methylated CpG tandems amplification and sequencing (MCTA-seq)

MCTA-seq is a novel DNA methylation analysis technique to detect hypermethylated CGIs in cfDNA (Wen et al., 2015). In this approach, bisulfite treatment is performed at first, and then semi-random primers are used to amplify the converted DNA at the 3'-end. Third, primers containing the CpG tandem sequence "CGCGCGG" at the 3'-end are used to selectively amplify the methylated CpG tandem sites. Only the pre-amplified sequences by the semi-random primer and "CGCGCGG" tandem primer can then be amplified and sequenced (Fig. 2). This approach can highly enrich CGIs in genome and solve the problem of low-input DNA through multiple rounds of amplification. Because it can only detect CpG tandem regions, MCTA-seq can decrease other methylation backgrounds.

3.4. Bisulfite-based methylation-specific region sequencing

Bisulfite-based methylation-specific region sequencing uses specific primers to amplify some interesting regions after bisulfite treatment (Fig. 2), such as using *SEPT9* promoter primers to diagnose lung cancer (Powrozek et al., 2014). A vital step for this method is how to design appropriate primers, as the complexity of DNA sequence would reduce after bisulfite treatment. There are software programs and guidelines which can be used to obtain available primers (Ostrow et al., 2010; Hernandez et al., 2013). Meanwhile, it is also essential to find the regions that can distinguish between tumor and normal tissues. This approach is one of the most convenient and cheap approaches for disease diagnosis. The simple procedure and rapid detection make this approach appropriate for clinical practice. However, there is also doubt about the accuracy of this approach, as cancer is heterogeneous and complicated.

3.5. 5hmC-seal technology for 5hmC profiling in cfDNA

Emerging evidence indicates that 5hmC not only marks active demethylation but also acts as a relatively stable epigenetic marker (Wu and Zhang, 2017). Recent studies reported that 5hmC has a correlation with gene regulation (Konstandin et al., 2011; Han et al., 2016), tumorigenesis (Haffner et al., 2011; Kraus et al., 2015) and other diseases (Barzilai et al., 2012). 5hmC displays a tissue-specific mass distribution and reduced 5hmC levels are also observed in solid tumors (Kriaucionis and Heintz, 2009; Globisch et al., 2010; Jin et al., 2011). These observations suggest that 5hmC signatures in cfDNA may also be potential biomarkers for cancer diagnostics. Recently, genome-wide profiling of 5hmC in cfDNA supports its role as markers for cancer diagnosis (Li et al., 2017; Song et al., 2017). The approach used in these two studies was optimized from a previously published hMe-Seal profiling method (Song et al., 2011), named 5hmC-Seal. In this approach, cfDNA is first ligated with sequencing adapters. Then, 5hmC-containing cfDNA fragments are labeled with a biotin group for pull-down with streptavidin beads.

Through the selective labeling reaction and subsequent pull-down procedure, 5hmC-containing cfDNA fragments can be specifically captured. The final library is amplified by PCR and then subject to high-throughput sequencing (Fig. 2).

The comparison of these cfDNA methylation analysis methods is summarized in Table 1, and the selection of these technologies mainly depends on the methylation changes to be detected. WGBS can detect DNA methylation changes in the all genome, while it is very expensive. scRRBS and MCTA-seq mainly detect methylation changes in the CGIs, the methylation states of which may have a correlation with gene expression. Bisulfite-based methylation-specific region sequencing can only detect methylation changes in specific regions and is mainly used to detect the methylation states of gene promoters. 5hmC-Seal is a technology to detect 5hmC changes.

4. Future directions

Proof-of-concept studies demonstrate that cfDNA methylation may be useful biomarkers and there are many prospective studies of the clinical utility of cfDNA methylation. However, a lot of challenges for cfDNA methylation analysis still remain, and the major challenge is that current approaches are essentially adapted to examining methylation in genomic DNA. Almost all of the cfDNA methylation analysis methods depend on bisulfite sequencing, which can cause a degree of DNA degradation. As cfDNA is highly fragmented and the quantity of cfDNA is limited, bisulfite treatment may lead to the loss of some DNA region and reduce the repeatability of the assay. WGBS provides whole methylation information in cfDNA, while this approach is expensive. Subsequent optimized approaches scRRBS and MCTA-seq aim at capturing CpG-enriched cfDNA fragments, which would lead to loss of some critical DNA methylation sites. More appropriate cfDNA methylation analysis approaches are needed to improve the sensitivity and specificity of cfDNA methylation biomarkers.

5hmC signatures in cfDNA have been reported as biomarkers for cancer diagnosis via 5hmC-Seal approach. However, the sensitivity of these 5hmC biomarkers may be limited as 5hmC-Seal cannot offer base resolution signal of 5hmC. Development of approaches, which can not only enrich 5hmC-containing cfDNA but also give the base resolution 5hmC information, will facilitate the clinical utility of 5hmC biomarkers in cfDNA. The other two DNA active demethylation intermediates 5fC and 5caC, although they present very low abundance in genome, are also potential functional epigenetic modifications. Future studies will test their potential as biomarkers in cfDNA.

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Table 1
Comparison of different cfDNA methylation analysis methods.

Analysis method	Advantage	Disadvantage
WGBS	Provide genome-wide methylation information at single-base resolution	High degree of DNA degradation and very expensive
scRRBS	Enriched CGIs and cost-effective	Further digestion of cfDNA leads to information lost
MCTA-seq	Highly sensitive methylation detection and cost-effective	The use of semi-random primers for amplification leads to information lost
Bisulfite-based methylation-specific region sequencing	Easy to perform and cost-effective	Only give information of specific regions and applicable to limited diseases
5hmC-Seal	Do not further degrade the highly fragmented cfDNA and enable cost-effective sequencing	Cannot provide single-base resolution information

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References

- Abdel-Wahab, O., Mullally, A., Hedvat, C., Garcia-Manero, G., Patel, J., Wadleigh, M., Malinge, S., Yao, J., Kilpivaara, O., Bhat, R., Huberman, K., Thomas, S., Dolgalev, I., Heguy, A., Paietta, E., Le Beau, M.M., Beran, M., Tallman, M.S., Ebert, B.L., Kantarjian, H.M., Stone, R.M., Gilliland, D.G., Crispino, J.D., Levine, R.L., 2009. Genetic characterization of TET1, TET2, and TET3 alterations in myeloid malignancies. *Blood* 114, 144–147.
- Allen, E.G., Freeman, S.B., Druschel, C., Hobbs, C.A., O'Leary, L.A., Romitti, P.A., Royle, M.H., Torfs, C.P., Sherman, S.L., 2009. Maternal age and risk for trisomy 21 assessed by the origin of chromosome nondisjunction: a report from the Atlanta and National Down Syndrome Projects. *Hum. Genet.* 125, 41–52.
- Barzilai, N., Huffman, D.M., Muzumdar, R.H., Bartke, A., 2012. The critical role of metabolic pathways in aging. *Diabetes* 61, 1315–1322.
- Baylin, S.B., Jones, P.A., 2011. A decade of exploring the cancer epigenome - biological and translational implications. *Nat. Rev. Cancer* 11, 726–734.
- Baylin, S.B., Jones, P.A., 2016. Epigenetic determinants of cancer. *Cold Spring Harb. Perspect. Biol.* 8, a019505.
- Bird, A., 2002. DNA methylation patterns and epigenetic memory. *Genes Dev.* 16, 6–21.
- Board, R.E., Knight, L., Greystoke, A., Blackhall, F.H., Hughes, A., Dive, C., Ranson, M., 2008. DNA methylation in circulating tumour DNA as a biomarker for cancer. *Biomark. Insights* 2, 307–319.
- Botezatu, I., 2000. Genetic analysis of DNA excreted in urine: a new approach for detecting specific genomic DNA sequences from cells dying in an organism. *Clin. Chem.* 46, 1078–1084.
- Branco, M.R., Ficiz, G., Reik, W., 2011. Uncovering the role of 5-hydroxymethylcytosine in the epigenome. *Nat. Rev. Genet.* 13, 7–13.
- Burnham, P., Khush, K., De Vlaminck, I., 2017. Myriad applications of circulating cell-free DNA in precision organ transplant monitoring. *Ann. Am. Thorac. Soc.* 14, S237–S241.
- Chan, K.C., Jiang, P., Chan, C.W., Sun, K., Wong, J., Hui, E.P., Chan, S.L., Chan, W.C., Hui, D.S., Ng, S.S., Chan, H.L., Wong, C.S., Ma, B.B., Chan, A.T., Lai, P.B., Sun, H., Chiu, R.W., Lo, Y.M., 2013. Noninvasive detection of cancer-associated genome-wide hypomethylation and copy number aberrations by plasma DNA bisulfite sequencing. *Proc. Natl. Acad. Sci. U. S. A.* 110, 18761–18768.
- Chan, K.C.A., Leung, S.F., Yeung, S.W., Chan, A.T.C., Lo, Y.M.D., 2008. Quantitative analysis of the transrenal excretion of circulating EBV DNA in nasopharyngeal carcinoma patients. *Clin. Cancer Res.* 14, 4809.
- Chim, S.S., Jin, S., Lee, T.Y., Lun, F.M., Lee, W.S., Chan, L.Y., Jin, Y., Yang, N., Tong, Y.K., Leung, T.Y., Lau, T.K., Ding, C., Chiu, R.W., Lo, Y.M., 2008. Systematic search for placental DNA-methylation markers on chromosome 21: toward a maternal plasma-based epigenetic test for fetal trisomy 21. *Clin. Chem.* 54, 500–511.
- Chim, S.S.C., Tong, Y.K., Chiu, R.W.K., Lau, T.K., Leung, T.N., Chan, L.Y.S., Oudejans, C.B.M., Ding, C., Lo, Y.M.D., 2005. Detection of the placental epigenetic signature of the maspin gene in maternal plasma. *Proc. Natl. Acad. Sci. U. S. A.* 102, 14753–14758.
- Cimmino, L., Abdel-Wahab, O., Levine, R.L., Aifantis, I., 2011. TET family proteins and their role in stem cell differentiation and transformation. *Cell Stem Cell* 9, 193–204.
- Dalton, S.R., Bellacosa, A., 2012. DNA demethylation by TDG. *Epigenomics* 4, 459–467.
- Daly, K.P., 2015. Circulating donor-derived cell-free DNA: a true biomarker for cardiac allograft rejection? *Ann. Transl. Med.* 3, 47.
- De Mattos-Arruda, L., 2015. Cerebrospinal fluid-derived circulating tumour DNA better represents the genomic alterations of brain tumours than plasma. *Nat. Commun.* 6, 8839.
- De Mattos-Arruda, L., Mayor, R., Ng, C.K., Weigelt, B., Martinez-Ricarte, F., Torrejon, D., Oliveira, M., Arias, A., Raventos, C., Tang, J., Guerini-Rocco, E., Martinez-Saez, E., Lois, S., Marin, O., de la Cruz, X., Piscuoglio, S., Towers, R., Vivancos, A., Peg, V., Ramon y Cajal, S., Carles, J., Rodon, J., Gonzalez-Cao, M., Taberero, J., Felipe, E., Sahuquillo, J., Berger, M.F., Cortes, J., Reis-Filho, J.S., Seoane, J., 2015. Cerebrospinal fluid-derived circulating tumour DNA better represents the genomic alterations of brain tumours than plasma. *Nat. Commun.* 6, 8839.
- De Mattos-Arruda, L., Weigelt, B., Cortes, J., Won, H.H., Ng, C.K., Nuciforo, P., Bidard, F.C., Aura, C., Saura, C., Peg, V., Piscuoglio, S., Oliveira, M., Smolders, Y., Patel, P., Norton, L., Taberero, J., Berger, M.F., Seoane, J., Reis-Filho, J.S., 2014. Capturing intra-tumor genetic heterogeneity by *de novo* mutation profiling of circulating cell-free tumor DNA: a proof-of-principle. *Ann. Oncol.* 25, 1729–1735.
- De Vlaminck, I., 2015. Noninvasive monitoring of infection and rejection after lung transplantation. *Proc. Natl. Acad. Sci. U. S. A.* 112, 13336–13341.
- Delhommeau, F., Dupont, S., Della Valle, V., James, C., Trannoy, S., Masse, A., Kosmider, O., Le Couedic, J.P., Robert, F., Alberdi, A., Lecluse, Y., Plo, I., Dreyfus, F.J., Marzac, C., Casadevall, N., Lacombe, C., Romana, S.P., Dessen, P., Soulier, J., Viguie, F., Fontenay, M., Vainchenker, W., Bernard, O.A., 2009. Mutation in *TET2* in myeloid cancers. *N. Engl. J. Med.* 360, 2289–2301.
- Diehl, F., Schmidt, K., Choti, M.A., Romans, K., Goodman, S., Li, M., Thornton, K., Agrawal, N., Sokoll, L., Szabo, S.A., Kinzler, K.W., Vogelstein, B.A., 2008. Circulating mutant DNA to assess tumor dynamics. *Nat. Med.* 14, 985–990.
- Esteller, M., 2007. Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat. Rev. Genet.* 8, 286–298.
- Feng, S., Jacobsen, S.E., Reik, W., 2010. Epigenetic reprogramming in plant and animal development. *Science* 330, 622–627.
- Fleischhacker, M., Schmidt, B., 2007. Circulating nucleic acids (CNAs) and cancer - a survey. *Biochim. Biophys. Acta* 1775, 181–232.
- Frommer, M., McDonald, L.E., Millar, D.S., Collis, C.M., Watt, F., Grigg, G.W., Molloy, P.L., Paul, C.L., 1992. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc. Natl. Acad. Sci. U. S. A.* 89, 1827–1831.
- Gadi, V.K., Nelson, J.L., Boespflug, N.D., Guthrie, K.A., Kuhr, C.S., 2006. Soluble donor DNA concentrations in recipient serum correlate with pancreas-kidney rejection. *Clin. Chem.* 52, 379–382.
- Garcia-Olmo, D.C., Dominguez, C., Garcia-Arranz, M., Anker, P., Stroun, M., Garcia-Verdugo, J.M., Garcia-Olmo, D., 2010. Cell-free nucleic acids circulating in the plasma of colorectal cancer patients induce the oncogenic transformation of susceptible cultured cells. *Cancer Res.* 70, 560–567.
- Globisch, D., Munzel, M., Muller, M., Michalakis, S., Wagner, M., Koch, S., Bruckl, T., Biel, M., Carell, T., 2010. Tissue distribution of 5-hydroxymethylcytosine and search for active demethylation intermediates. *PLoS One* 5, e15367.
- Gold, B., Cankovic, M., Furtado, L.V., Meier, F., Gocke, C.D., 2015. Do circulating tumor cells, exosomes, and circulating tumor nucleic acids have clinical utility? A report of the association for molecular pathology. *J. Mol. Diagn.* 17, 209–224.
- Gould, T.J., Lysov, Z., Liaw, P.C., 2015. Extracellular DNA and histones: double-edged swords in immunothrombosis. *J. Thromb. Haemostasis* 13 (Suppl. 1), S82–S91.
- Guibert, J., Benachi, A., Grebille, A.G., Ernault, P., Zorn, J.R., Costa, J.M., 2003. Kinetics of *SKY* gene appearance in maternal serum: detection by real time PCR in early pregnancy after assisted reproductive technique. *Hum. Reprod.* 18, 1733–1736.
- Guo, H., Zhu, P., Wu, X., Li, X., Wen, L., Tang, F., 2013. Single-cell methylome landscapes of mouse embryonic stem cells and early embryos analyzed using reduced representation bisulfite sequencing. *Genome Res.* 23, 2126–2135.
- Guo, S., Diep, D., Plongthongkum, N., Fung, H.L., Zhang, K., Zhang, K., 2017. Identification of methylation haplotype blocks aids in deconvolution of heterogeneous tissue samples and tumor tissue-of-origin mapping from plasma DNA. *Nat. Genet.* 49, 635–642.
- Haffner, M.C., Chaux, A., Meeker, A.K., Esopi, D.M., Gerber, J., Pellakuru, L.G., Toubaji, A., Argani, P., Iacobuzio-Donahue, C., Nelson, W.G., Netto, G.J., De Marzo, A.M., Yegnasubramanian, S., 2011. Global 5-hydroxymethylcytosine content is significantly reduced in tissue stem/progenitor cell compartments and in human cancers. *Oncotarget* 2, 627–637.
- Han, D., Lu, X., Shih, A.H., Nie, J., You, Q., Xu, M.M., Melnick, A.M., Levine, R.L., He, C., 2016. A highly sensitive and robust method for genome-wide 5hmC profiling of rare cell populations. *Mol. Cell* 63, 711–719.
- He, Y.F., Li, B.Z., Li, Z., Liu, P., Wang, Y., Tang, Q., Ding, J., Jia, Y., Chen, Z., Li, L., Sun, Y., Li, X., Dai, Q., Song, C.X., Zhang, K., He, C., Xu, G.L., 2011. Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. *Science* 333, 1303–1307.
- Hernandez, H.G., Tse, M.Y., Pang, S.C., Arboleda, H., Forero, D.A., 2013. Optimizing methodologies for PCR-based DNA methylation analysis. *Biotechniques* 55, 181–197.
- Hsu, C.H., Peng, K.L., Kang, M.L., Chen, Y.R., Yang, Y.C., Tsai, C.H., Chu, C.S., Jeng, Y.M., Chen, Y.T., Lin, F.M., Huang, H.D., Lu, Y.Y., Teng, Y.C., Lin, S.T., Lin, R.K., Tang, F.M., Lee, S.B., Hsu, H.M., Yu, J.C., Hsiao, P.W., Juan, L.J., 2012. TET1 suppresses cancer invasion by activating the tissue inhibitors of metalloproteinases. *Cell Rep.* 2, 568–579.
- Irizarry, R.A., Ladd-Acosta, C., Wen, B., Wu, Z., Montano, C., Onyango, P., Cui, H., Gabo, K., Rongione, M., Webster, M., Ji, H., Potash, J., Sabuncyan, S., Feinberg, A.P., 2009. The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. *Nat. Genet.* 41, 178–186.
- Ito, S., D'Alessio, A.C., Taranova, O.V., Hong, K., Sowers, L.C., Zhang, Y., 2010. Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature* 466, 1129–1133.
- Jaenisch, R., Bird, A., 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat. Genet.* 33 (Suppl. 1), 245–254.
- Jahr, S., 2001. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res.* 61, 1659–1665.
- Jamal-Hanjani, M., Wilson, G.A., Horswell, S., Mitter, R., Sakarya, O., Constantin, T., Salari, R., Kirizilar, E., Sigurdsson, S., Pelham, R., Kareh, S., Zimmermann, B., Swanton, C., 2016. Detection of ubiquitous and heterogeneous mutations in cell-free DNA from patients with early-stage non-small-cell lung cancer. *Ann. Oncol.* 27, 862–867.
- Jin, S.G., Jiang, Y., Qiu, R., Rauch, T.A., Wang, Y., Schackert, G., Krex, D., Lu, Q., Pfeifer, G.P., 2011. 5-Hydroxymethylcytosine is strongly depleted in human cancers but its levels do not correlate with *IDH1* mutations. *Cancer Res.* 71, 7360–7365.
- Jones, P.A., 2012. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat. Rev. Genet.* 13, 484–492.
- Jung, K., Fleischhacker, M., Rabien, A., 2010. Cell-free DNA in the blood as a solid tumor biomarker-a critical appraisal of the literature. *Clin. Chim. Acta* 411, 1611–1624.
- Kaplan, M.J., Radic, M., 2012. Neutrophil extracellular traps: double-edged swords

- of innate immunity. *J. Immunol.* 189, 2689–2695.
- Kinnings, S.L., Geis, J.A., Almasri, E., Wang, H., Guan, X., McCullough, R.M., Bombard, A.T., Saldívar, J.S., Oeth, P., Deciu, C., 2015. Factors affecting levels of circulating cell-free fetal DNA in maternal plasma and their implications for noninvasive prenatal testing. *Prenat. Diagn.* 35, 816–822.
- Konstandin, N., Bultmann, S., Swagierczak, A., Dufour, A., Ksienzyk, B., Schneider, F., Herold, T., Mulaw, M., Kakadia, P.M., Schneider, S., Spiekermann, K., Leonhardt, H., Bohlander, S.K., 2011. Genomic 5-hydroxymethylcytosine levels correlate with *TET2* mutations and a distinct global gene expression pattern in secondary acute myeloid leukemia. *Leukemia* 25, 1649–1652.
- Kraus, T.F., Guibourt, V., Kretschmar, H.A., 2015. 5-Hydroxymethylcytosine, the “Sixth Base”, during brain development and ageing. *J. Neural Transm. (Vienna)* 122, 1035–1043.
- Kriaucionis, S., Heintz, N., 2009. The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. *Science* 324, 929–930.
- Legendre, C., Gooden, G.C., Johnson, K., Martinez, R.A., Liang, W.S., Sallia, B., 2015. Whole-genome bisulfite sequencing of cell-free DNA identifies signature associated with metastatic breast cancer. *Clin. Epigenetics* 7, 100.
- Lehmann-Werman, R., Neiman, D., Zemmour, H., Moss, J., Magenheimer, J., Vaknin-Dembinsky, A., Rubertsson, S., Nellgard, B., Blennow, K., Zetterberg, H., Spalding, K., Haller, M.J., Wasserfall, C.H., Schatz, D.A., Greenbaum, C.J., Dorrell, C., Grompe, M., Zick, A., Hubert, A., Mao, M., Fendrich, V., Bartsch, D.K., Golan, T., Ben Sasson, S.A., Zamir, G., Razin, A., Cedar, H., Shapiro, A.M., Glaser, B., Shemer, R., Dor, Y., 2016. Identification of tissue-specific cell death using methylation patterns of circulating DNA. *Proc. Natl. Acad. Sci. U. S. A.* 113, E1826–E1834.
- Leon, S.A., Shapiro, B., Sklaroff, D.M., Yaros, M.J., 1977. Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res.* 37, 646–650.
- Li, W., Zhang, X., Lu, X., You, L., Song, Y., Luo, Z., Zhang, J., Nie, J., Zheng, W., Xu, D., Wang, Y., Dong, Y., Yu, S., Hong, J., Shi, J., Hao, H., Luo, F., Hua, L., Wang, P., Qian, X., Yuan, F., Wei, L., Cui, M., Zhang, T., Liao, Q., Dai, M., Liu, Z., Chen, G., Meckel, K., Adhikari, S., Jia, G., Bissonnette, M.B., Zhang, X., Zhao, Y., Zhang, W., He, C., Liu, J., 2017. 5-Hydroxymethylcytosine signatures in circulating cell-free DNA as diagnostic biomarkers for human cancers. *Cell Res.* 27, 1243–1257.
- Lian, C.G., Xu, Y., Ceol, C., Wu, F., Larson, A., Dresser, K., Xu, W., Tan, L., Hu, Y., Zhan, Q., Lee, C.W., Hu, D., Lian, B.Q., Kleffel, S., Yang, Y., Neiswender, J., Khorasani, A.J., Fang, R., Lezcano, C., Duncan, L.M., Scolyer, R.A., Thompson, J.F., Kakavand, H., Houvras, Y., Zon, L.I., Mihm Jr., M.C., Kaiser, U.B., Schatton, T., Woda, B.A., Murphy, G.F., Shi, Y.G., 2012. Loss of 5-hydroxymethylcytosine is an epigenetic hallmark of melanoma. *Cell* 150, 1135–1146.
- Lissa, D., Robles, A.J., 2016. Methylation analyses in liquid biopsy. *Transl. Lung Cancer Res.* 5, 492–504.
- Lister, R., O'Malley, R.C., Tonti-Filippini, J., Gregory, B.D., Berry, C.C., Millar, A.H., Ecker, J.R., 2008. Highly integrated single-base resolution maps of the epigenome in *Arabidopsis*. *Cell* 133, 523–536.
- Lister, R., Pelizzola, M., Dowen, R.H., Hawkins, R.D., Hon, G., Tonti-Filippini, J., Nery, J.R., Lee, L., Ye, Z., Ngo, Q.M., Edsall, L., Antosiewicz-Bourget, J., Stewart, R., Ruotti, V., Millar, A.H., Thomson, J.A., Ren, B., Ecker, J.R., 2009. Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature* 462, 315–322.
- Lo, Y.M., 1999. Rapid clearance of fetal DNA from maternal plasma. *Am. J. Hum. Genet.* 64, 218–224.
- Lo, Y.M., Chan, K.C., Sun, H., Chen, E.Z., Jiang, P., Lun, F.M., Zheng, Y.W., Leung, T.Y., Lau, T.K., Cantor, C.R., Chiu, R.W., 2010. Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus. *Sci. Transl. Med.* 2, 61ra91.
- Lo, Y.M.D., 1997. Presence of fetal DNA in maternal plasma and serum. *Lancet* 350, 485–487.
- Lo, Y.M.D., 2010. Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus. *Sci. Transl. Med.* 2, 61ra91.
- Lo, Y.M.D., Tein, M.S.C., Pang, C.C.P., Yeung, C.K., Tong, K.L., 1998. Presence of donor-specific DNA in plasma of kidney and liver-transplant recipients. *Lancet* 351, 1329–1330.
- Lui, Y.Y., Woo, K.S., Wang, A.Y., Yeung, C.K., Li, P.K., Chau, E., Ruygrok, P., Lo, Y.M., 2003. Origin of plasma cell-free DNA after solid organ transplantation. *Clin. Chem.* 49, 495–496.
- Lun, F.M., Chiu, R.W., Sun, K., Leung, T.Y., Jiang, P., Chan, K.C., Sun, H., Lo, Y.M., 2013. Noninvasive prenatal methylation analysis by genome-wide bisulfite sequencing of maternal plasma DNA. *Clin. Chem.* 59, 1583–1594.
- Maiti, A., Drohat, A.C., 2011. Thymine DNA glycosylase can rapidly excise 5-formylcytosine and 5-carboxylcytosine: potential implications for active demethylation of CpG sites. *J. Biol. Chem.* 286, 35334–35338.
- Mandel, P., Metais, P., 1948. Les acides nucléiques du plasma sanguin chez l'homme. *C. R. Acad. Sci. Paris* 142, 241–243.
- Meissner, A., Mikkelsen, T.S., Gu, H., Wernig, M., Hanna, J., Sivachenko, A., Zhang, X., Bernstein, B.E., Nusbaum, C., Jaffe, D.B., Gnirke, A., Jaenisch, R., Lander, E.S., 2008. Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature* 454, 766–770.
- Mithani, S.K., 2007. Mitochondrial resequencing arrays detect tumor-specific mutations in salivary rinses of patients with head and neck cancer. *Clin. Cancer Res.* 13, 7335–7340.
- Moreira, V.G., Garca, B.P., Martn, J.M.B., Surez, F.O., Alvarez, F.V., 2009. Cell-free DNA as a noninvasive acute rejection marker in renal transplantation. *Clin. Chem.* 55, 1958–1966.
- Mouliere, F., El Messaoudi, S., Pang, D., Dritschilo, A., Thierry, A.R., 2014. Multi-marker analysis of circulating cell-free DNA toward personalized medicine for colorectal cancer. *Mol. Oncol.* 8, 927–941.
- Mouliere, F., Robert, B., Arnau Peyrotte, E., Del Rio, M., Ychou, M., Molina, F., Gongora, C., Thierry, A.R., 2011. High fragmentation characterizes tumour-derived circulating DNA. *PLoS One* 6, e23418.
- Ng, E.K., Tsui, N.B., Lau, T.K., Leung, T.N., Chiu, R.W., Panesar, N.S., Lit, L.C., Chan, K.W., Lo, Y.M., 2003. mRNA of placental origin is readily detectable in maternal plasma. *Proc. Natl. Acad. Sci. U. S. A.* 100, 4748–4753.
- Niu, Q., Li, X., Xia, D., Jiang, Y., Tian, Z., Bian, C., Zhang, C., Liu, P., Zhang, F., Yang, Y., Wang, G., 2017. MicroRNA-186 affects the proliferation of tumor cells via yes-associated protein 1 in the occurrence and development of pancreatic cancer. *Exp. Ther. Med.* 14, 2094–2100.
- Old, R.W., Crea, F., Puszyk, W., Hulten, M.A., 2007. Candidate epigenetic biomarkers for non-invasive prenatal diagnosis of Down syndrome. *Reprod. Biomed. Online* 15, 227–235.
- Ono, R., Taki, T., Taketani, T., Taniwaki, M., Kobayashi, H., Hayashi, Y., 2002. LCX, leukemia-associated protein with a CXXC domain, is fused to MLL in acute myeloid leukemia with trilineage dysplasia having t(10;11)(q22;q23). *Cancer Res.* 62, 4075–4080.
- Ostrow, K.L., Hoque, M.O., Loyo, M., Brait, M., Greenberg, A., Siegfried, J.M., Grandis, J.R., 2010. Molecular analysis of plasma DNA for the early detection of lung cancer by quantitative methylation-specific PCR. *Clin. Cancer Res.* 16, 3463–3472.
- Pan, W., Gu, W., Nagpal, S., Gephart, M.H., Quake, S.R., 2015. Brain tumor mutations detected in cerebral spinal fluid. *Clin. Chem.* 61, 514–522.
- Pfaffeneder, T., Hackner, B., Truss, M., Munzel, M., Muller, M., Deiml, C.A., Hagemeyer, C., Carell, T., 2011. The discovery of 5-formylcytosine in embryonic stem cell DNA. *Angew. Chem. Int. Ed. Engl.* 50, 7008–7012.
- Poon, L.L.M., Leung, T.N., Lau, T.K., Chow, K.C.K., Lo, Y.M.D., 2002. Differential DNA methylation between fetus and mother as a strategy for detecting fetal DNA in maternal plasma. *Clin. Chem.* 48, 35–41.
- Powrozok, T., Krawczyk, P., Kucharczyk, T., Milanowski, J., 2014. Septin 9 promoter methylation in free circulating DNA-potential role in noninvasive diagnosis of lung cancer: preliminary report. *Med. Oncol.* 31, 917.
- Rodrigues Filho, E.M., 2014. Elevated cell-free plasma DNA level as an independent predictor of mortality in patients with severe traumatic brain injury. *J. Neurotrauma* 31, 1639–1646.
- Schitz, E., Fischer, A., Beck, J., Harden, M., Koch, M., Wuensch, T., Stockmann, M., Nashed, B., Kollmar, O., Matthaei, J., Kanzow, P., Walson, P.D., Brockmiller, J., Oellerich, M., 2017. Graft-derived cell-free DNA, a noninvasive early rejection and graft damage marker in liver transplantation: a prospective, observational, multicenter cohort study. *PLoS Med.* 14, e1002286.
- Schwarzenbach, H., Hoon, D.S., Pantel, K., 2011. Cell-free nucleic acids as biomarkers in cancer patients. *Nat. Rev. Cancer* 11, 426–437.
- Smith, Z.D., Meissner, A., 2013. DNA methylation: roles in mammalian development. *Nat. Rev. Genet.* 14, 204–220.
- Snyder, T.M., Khush, K.K., Valentine, H.A., Quake, S.R., 2011. Universal noninvasive detection of solid organ transplant rejection. *Proc. Natl. Acad. Sci. U. S. A.* 108, 6229–6234.
- Song, C.X., Szulwach, K.E., Fu, Y., Dai, Q., Yi, C., Li, X., Li, Y., Chen, C.H., Zhang, W., Jian, X., Wang, J., Zhang, L., Looney, T.J., Zhang, B., Godley, L.A., Hicks, L.M., Lahn, B.T., Jin, P., He, C., 2011. Selective chemical labeling reveals the genome-wide distribution of 5-hydroxymethylcytosine. *Nat. Biotechnol.* 29, 68–72.
- Song, C.X., Yin, S., Ma, L., Wheeler, A., Chen, Y., Zhang, Y., Liu, B., Xiong, J., Zhang, W., Hu, J., Zhou, Z., Dong, B., Tian, Z., Jeffrey, S.S., Chua, M.S., So, S., Li, W., Wei, Y., Diao, J., Xie, D., Quake, S.R., 2017. 5-Hydroxymethylcytosine signatures in cell-free DNA provide information about tumor types and stages. *Cell Res.* 27, 1231–1242.
- Sriram, K.B., Relan, V., Clarke, B.E., Duhig, E.E., Windsor, M.N., Matar, K.S., Naidoo, R., Passmore, L., McCaul, E., Courtney, D., Yang, I.A., Bowman, R.V., Fong, K.M., 2012. Pleural fluid cell-free DNA integrity index to identify cytologically negative malignant pleural effusions including mesotheliomas. *BMC Cancer* 12, 428.
- Stroun, M., Anker, P., Maurice, P., Lyautey, J., Lederrey, C., Beljanski, M., 1989. Neoplastic characteristics of the DNA found in the plasma of cancer patients. *Oncology* 46, 318–322.
- Stroun, M., Maurice, P., Vasioukhin, V., Lyautey, J., Lederrey, C., Lefort, F., Rossier, A., Chen, X.Q., Anker, P., 2000. The origin and mechanism of circulating DNA. *Ann. N. Y. Acad. Sci.* 906, 161–168.
- Sun, K., Jiang, P., Chan, K.C., Wong, J., Cheng, Y.K., Liang, R.H., Chan, W.K., Ma, E.S., Chan, S.L., Cheng, S.H., Chan, R.W., Tong, Y.K., Ng, S.S., Wong, R.S., Hui, D.S., Leung, T.N., Leung, T.Y., Lai, P.B., Chiu, R.W., Lo, Y.M., 2015. Plasma DNA tissue mapping by genome-wide methylation sequencing for noninvasive prenatal, cancer, and transplantation assessments. *Proc. Natl. Acad. Sci. U. S. A.* 112, E5503–E5512.
- Tahiliani, M., Koh, K.P., Shen, Y., Pastor, W.A., Bandukwala, H., Brudno, Y., Agarwal, S., Iyer, L.M., Liu, D.R., Aravind, L., Rao, A., 2009. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* 324, 930–935.
- Tanaka, K., Okamoto, A., 2007. Degradation of DNA by bisulfite treatment. *Bioorg. Med. Chem. Lett.* 17, 1912–1915.
- Tefferi, A., Lim, K.H., Levine, R., 2009. Mutation in *TET2* in myeloid cancers. *N. Engl. J. Med.* 361, 1117 author reply 1117–1118.
- Thienpont, B., Steinbacher, J., Zhao, H., D'Anna, F., Kuchnio, A., Ploumakis, A., Ghesquiere, B., Van Dyck, L., Boeckx, B., Schoonjans, L., Hermans, E., Amant, F., Kristensen, V.N., Peng Koh, K., Mazzone, M., Coleman, M., Carell, T., Carmeliet, P.,

- Lambrechts, D., 2016. Tumour hypoxia causes DNA hypermethylation by reducing TET activity. *Nature* 537, 63–68.
- Thierry, A.R., 2010. Origin and quantification of circulating DNA in mice with human colorectal cancer xenografts. *Nucleic Acids Res.* 38, 6159–6175.
- To, E.W.H., 2003. Rapid clearance of plasma Epstein-Barr virus DNA after surgical treatment of nasopharyngeal carcinoma. *Clin. Cancer Res.* 9, 3254–3259.
- Tong, Y.K., Ding, C., Chiu, R.W., Gerovassili, A., Chim, S.S., Leung, T.Y., Leung, T.N., Lau, T.K., Nicolaides, K.H., Lo, Y.M., 2006. Noninvasive prenatal detection of fetal trisomy 18 by epigenetic allelic ratio analysis in maternal plasma: theoretical and empirical considerations. *Clin. Chem.* 52, 2194–2202.
- Trejo-Becerril, C., Perez-Cardenas, E., Taja-Chayeb, L., Anker, P., Herrera-Goepfert, R., Medina-Velazquez, L.A., Hidalgo-Miranda, A., Perez-Montiel, D., Chavez-Blanco, A., Cruz-Velazquez, J., Diaz-Chavez, J., Gaxiola, M., Duenas-Gonzalez, A., 2012. Cancer progression mediated by horizontal gene transfer in an *in vivo* model. *PLoS One* 7, e52754.
- Wan, J.C.M., Massie, C., Garcia-Corbacho, J., Mouliere, F., Brenton, J.D., Caldas, C., Pacey, S., Baird, R., Rosenfeld, N., 2017. Liquid biopsies come of age: towards implementation of circulating tumour DNA. *Nat. Rev. Cancer* 17, 223–238.
- Wang, E., Batey, A., Struble, C., Musci, T., Song, K., Oliphant, A., 2013. Gestational age and maternal weight effects on fetal cell-free DNA in maternal plasma. *Prenat. Diagn.* 33, 662–666.
- Wang, Y., 2015. Detection of tumor-derived DNA in cerebrospinal fluid of patients with primary tumors of the brain and spinal cord. *Proc. Natl. Acad. Sci. U. S. A.* 112, 9704–9709.
- Wang, Y., Chen, M., Xiao, N., Liu, H., 2016. Evaluation and comparison of *in vitro* degradation kinetics of DNA in serum, urine and saliva: a qualitative study. *Gene* 590, 142–148.
- Wen, L., Li, J., Guo, H., Liu, X., Zheng, S., Zhang, D., Zhu, W., Qu, J., Guo, L., Du, D., Jin, X., Zhang, Y., Gao, Y., Shen, J., Ge, H., Tang, F., Huang, Y., Peng, J., 2015. Genome-scale detection of hypermethylated CpG islands in circulating cell-free DNA of hepatocellular carcinoma patients. *Cell Res.* 25, 1376.
- Wu, H., D'Alessio, A.C., Ito, S., Wang, Z., Cui, K., Zhao, K., Sun, Y.E., Zhang, Y., 2011. Genome-wide analysis of 5-hydroxymethylcytosine distribution reveals its dual function in transcriptional regulation in mouse embryonic stem cells. *Genes Dev.* 25, 679–684.
- Wu, X., Zhang, Y., 2017. TET-mediated active DNA demethylation: mechanism, function and beyond. *Nat. Rev. Genet.* 18, 517–534.
- Xu, R.H., Wei, W., Krawczyk, M., Wang, W., Luo, H., Flagg, K., Yi, S., Shi, W., Quan, Q., Li, K., Zheng, L., Zhang, H., Caughey, B.A., Zhao, Q., Hou, J., Zhang, R., Xu, Y., Cai, H., Li, G., Hou, R., Zhong, Z., Lin, D., Fu, X., Zhu, J., Duan, Y., Yu, M., Ying, B., Zhang, W., Wang, J., Zhang, E., Zhang, C., Li, O., Guo, R., Carter, H., Zhu, J.K., Hao, X., Zhang, K., 2017. Circulating tumour DNA methylation markers for diagnosis and prognosis of hepatocellular carcinoma. *Nat. Mater.* 16, 1155–1161.
- Yang, H., Liu, Y., Bai, F., Zhang, J.Y., Ma, S.H., Liu, J., Xu, Z.D., Zhu, H.G., Ling, Z.Q., Ye, D., Guan, K.L., Xiong, Y., 2013. Tumor development is associated with decrease of *TET* gene expression and 5-methylcytosine hydroxylation. *Oncogene* 32, 663–669.
- Zimmermann, B., Hill, M., Gemelos, G., Demko, Z., Banjevic, M., Baner, J., Ryan, A., Sigurjonsson, S., Chopra, N., Dodd, M., Levy, B., Rabinowitz, M., 2012. Noninvasive prenatal aneuploidy testing of chromosomes 13, 18, 21, X, and Y, using targeted sequencing of polymorphic loci. *Prenat. Diagn.* 32, 1233–1241.