Circulating cell-free DNA-based methylation pattern in plasma for early diagnosis of esophagus cancer

Rui Wang[1], Yue Yang[2], Tianyu Lu[2], Youbin Cui[2], Bo Li[1] and Xin Liu[2]

[1] School of Public Health, Jilin University, Changchun, Jilin, China
[2] Department of Thoracic Surgery, First Hospital of Jilin University, Changchun, Jilin, China

ABSTRACT

With the increased awareness of early tumor detection, the importance of detecting and diagnosing esophageal cancer in its early stages has been underscored. Studies have consistently demonstrated the crucial role of methylation levels in circulating cell-free DNA (cfDNA) in identifying and diagnosing early-stage cancer. cfDNA methylation pertains to the methylation state within the genomic scope of cfDNA and is strongly associated with cancer development and progression. Several research teams have delved into the potential application of cfDNA methylation in identifying early-stage esophageal cancer and have achieved promising outcomes. Recent research supports the high sensitivity and specificity of cfDNA methylation in early esophageal cancer diagnosis, providing a more accurate and efficient approach for early detection and improved clinical management. Accordingly, this review aims to present an overview of methylation-based cfDNA research with a focus on the latest developments in the early detection of esophageal cancer. Additionally, this review summarizes advanced analytical technologies for cfDNA methylation that have significantly benefited from recent advancements in separation and detection techniques, such as methylated DNA immunoprecipitation sequencing (MeDIP-seq). Recent findings suggest that biomarkers based on cfDNA methylation may soon find successful applications in the early detection of esophageal cancer. However, large-scale prospective clinical trials are required to identify the potential of these biomarkers.

INTRODUCTION

Esophageal cancer (EC) is a globally prevalent malignancy. According to the GLOBOCAN (2020) data (*Sung et al., 2021*), EC ranks eighth in terms of incidence and sixth in terms of cancer-related mortality. Predictions based on GLOBOCAN (2020) data by *Morgan et al. (2022)* suggest that there will be 957,000 new cases and 880,000 deaths from EC in 2,040. Developing countries account for approximately 80% of new EC cases, with 60% occurring in China (*Ferlay et al., 2010*). China is a high-risk country for EC (*Zhao et al., 2012*; *Chen et al., 2016*), with an estimated 480,000 new cases and 370,000 deaths annually (*Zhou et al., 2019*). Age and regional differentiation are the most significant characteristics of EC in China, with a higher incidence among males and in rural areas (*Liang, Fan &*

Qiao, 2017; *Chen et al., 2023*). Due to delayed diagnosis, surgical resection often cannot provide a cure. In China, the survival rate of patients with late-stage esophageal squamous cell carcinoma (ESCC) is less than 10%, while early detection leads to an 85% survival rate (*Wang et al., 2004*). However, cancer screening still relies on non-molecular techniques with low specificity and sensitivity. Currently, esophageal mucosal iodine (Lugol's) staining endoscopy is the gold standard for diagnosing abnormal esophageal mucosal lesions (*Liang, Fan & Qiao, 2017*; *Piñerúa Gonsálvez, Zambrano-Infantino & Benítez, 2019*). Liquid biopsy for tumors, with its simple procedure, high sensitivity and specificity, and noninvasive or minimally invasive features, has revolutionized traditional cancer treatment by dynamically monitoring the onset, development, and metastasis of the disease by detecting relevant tumor markers in patients' blood (*Peneder et al., 2021*).

Numerous studies have demonstrated the potential of circulating cell-free DNA (cfDNA) as a biomarker for early cancer diagnosis, making it a valuable liquid biopsy analyte (*Freitas et al., 2021*; *Jiang et al., 2022*). For instance, *Egyud et al. (2019)* showed the dynamic potential of cfDNA as a biomarker for monitoring treatment response and disease recurrence in patients with esophageal adenocarcinoma (EDAC). Likewise, *Azad et al. (2020)* found that cfDNA in blood samples from patients undergoing chemoradiotherapy for EC was associated with tumor progression, metastasis, and disease-specific survival. The methylation features of cfDNA are critical epigenetic modifications that exhibit significant differences between healthy individuals and those with various diseases, particularly malignant tumors (*Klein et al., 2021*). Moreover, prospective studies, such as Circulating Cell-Free Genome Atlas (CCGA) conducted by GRAIL (NCT02889978 and NCT3085888) confirmed the high specificity and sensitivity of cfDNA methylation for the early detection of various cancers (*Liu et al., 2020a*).

Numerous scholars have reviewed the applications of cfDNA in the early diagnosis of various tumors, such as central nervous system tumors and head and neck cancers (*McEwe, Leary & Lockwood, 2020*; *Birknerova et al., 2022*). Furthermore, some researchers have meticulously summarized the applications of cfDNA in directing adjuvant therapy for EC (*Salati et al., 2021*). However, there remains a gap in this field, as only a few scholars have explored the potential applications of cfDNA methylation in the early diagnosis of esophageal cancer. This article aims to review the latest advancement in cfDNA detection and its application in the early detection of EC. First, the biological characteristics of cfDNA are outlined. Then, the latest research on cfDNA methylation biomarkers for early detection of EC is summarized, along with the currently available sequencing methods for cfDNA methylation. Finally, the clinical utility, limitations, and future development directions of cfDNA in the early detection of EC are discussed.

SURVEY METHODOLOGY

The PubMed database was utilized to conduct a literature search related to the keywords "cell-free DNA", "circulating tumor DNA", "DNA methylation", "esophageal cancer", "cancer", and "early diagnosis". Subsequently, we collated the retrieved articles, including those cited in the recovered articles. Approximately 1,100 related articles were thoroughly read between January 2018 and May 2022.

The rationale for why it is needed

Numerous studies have extensively reviewed the application of cfDNA in the early diagnosis of various tumors, including liver and lung cancers. Furthermore, some researchers have provided comprehensive summaries of the use of cfDNA as adjuvant therapy for esophageal cancer. However, insufficient evidence exists as only a few scholars have reviewed the potential applications of cfDNA methylation in the early diagnosis of EC.

This review aims to provide a comprehensive overview of methylation-based cfDNA research and the latest advancements in the early detection of EC. Thus, researchers can gain a preliminary understanding of the relevance of cfDNA methylation and its application in the early detection of EC. Finally, we address the clinical applications, limitations, and future directions of cfDNA in the early detection of EC. This enables readers to comprehend the limitations and potential areas for further development in this research field, thereby promoting significant advancements in scientific achievements within this domain.

The audience it is intended for

Doctors specializing in oncology, thoracic surgery, and clinical laboratory medicine may find this study intriguing. The integration of cfDNA methylation with machine learning has accelerated the transition of this field into clinical applications. Ultimately, as cfDNA detection technology continues to advance and becomes implemented in clinical practice, patients can anticipate reduced treatment costs and significantly improved survival rates.

cfDNA and ctDNA biology

cfDNA refers to fragments of chromosomal material released due to cell death and is present in the circulatory system (*Lo et al., 2010*; *Heitzer, Auinger & Speicher, 2020*). Typically, these double-stranded fragments are approximately 150–200 base pairs in length (*Warton et al., 2014*). In healthy individuals, cfDNA mainly originates from hematopoietic cells; however, its composition may change under certain physiological or pathological conditions. This difference has been exploited for noninvasive liquid biopsy, with fetal-specific cfDNA used for prenatal diagnosis (*Lo et al., 1997*; *Chiu et al., 2011*; *Hou et al., 2012*) and tumor-specific cfDNA used for cancer diagnosis at different stages (*Abbosh et al., 2017*; *Dasari et al., 2020*; *Nakamura et al., 2021*; *Herberts et al., 2022*). Under normal physiological conditions, cfDNA concentrations in healthy individuals typically range from 1–50 ng/mL, whereas cfDNA concentrations in patients with tumors can exceed 1,000 ng/mL (*Meddeb et al., 2019*; *Osumi et al., 2019*). The genetic information contained in cfDNA can accurately detect tumor mutations and gene expressions (*Esfahani et al., 2022*), and its short half-life (16 min to 2 h) enables dynamic tracking of cancer progression (*Lo et al., 1999*; *Yu et al., 2013*).

ctDNA, released by tumor cells, is a type of cfDNA biomarker that carries tumor-specific genetic and epigenetic abnormalities. It serves as a potential substitute for tumor tissue DNA for diagnosing and monitoring prognostic changes (*Jin et al., 2020*; *Yang et al., 2020*; *Zhou et al., 2021*; *Li et al., 2022*). However, despite the promise of ctDNA mutation detection, its sensitivity and accuracy in detecting early-stage cancer remain low (*Cohen et al., 2018*). Abnormal changes in DNA methylation, including high CpG

island (CGI) methylation (*Weinberg et al., 2021*; *Ren et al., 2022*), are among the earliest molecular changes in cancer progression. *Loyfer et al. (2023)* recently published the most comprehensive human single-cell-type DNA methylation atlas to date, showing remarkable consistency in the DNA methylation patterns of the same cell type among different healthy individuals. Based on the highly conserved DNA methylation patterns of the same cell type among individuals, *Loyfer et al. (2023)* studied cell-specific differentially methylated regions to detect the content of specific cell types within cfDNA mixtures. Several studies have examined early cancer detection based on ctDNA methylation features (*Luo et al., 2020*; *Papanicolau-Sengos & Aldape, 2022*).

The final concentration of ctDNA can be influenced by several factors, such as tumor volume, location, and vascular formation, as well as anti-tumor treatments, such as surgery, chemotherapy, and radiotherapy. Liver and kidney clearance rates also affect ctDNA concentration (*Bettegowda et al., 2014*). Moreover, ctDNA concentration can be affected by other conditions such as trauma, myocardial infarction, stroke, and chronic diseases such as diabetes and inflammation. Optimal fluid selection also plays a crucial role in improving ctDNA detection. For instance, monitoring ctDNA in cerebrospinal fluid can reflect the disease status of patients with brain metastases (*Wu et al., 2023*), and using urine can facilitate noninvasive liver cancer screening (*Kim et al., 2022*).

Given the complexity of cfDNA and ctDNA, several factors must be considered when conducting liquid biopsy-related research. In addition, various techniques and detection instruments, along with pre-analytical factors, are essential for the comprehensive and accurate detection and analysis of all circulating DNA.

Pre-analytical and analytical phase examination

The pre-analytical workflow begins with the selection of an appropriate sample type, with plasma being a clinically convenient and compliant option. Currently, blood-based detection is the standard for multiple cancers (*Abbosh, Birkbak & Swanton, 2018*; *Luo et al., 2020*; *Ignatiadis, Sledge & Jeffrey, 2021*). However, there are several factors to consider during the pre-analysis of ctDNA, such as blood vessel selection, processing delay, centrifugation protocols, sample transportation, storage conditions, and anticoagulant selection, all of which affect the concentration of ctDNA (*Jen, Wu & Sidransky, 2000*; *Sozzi et al., 2005*; *Leest et al., 2020*; *Lehle et al., 2023*). In 2022, European Society for Medical Oncology (EMSO) (*Pascual et al., 2022*) released recommendations for ctDNA detection technology that include the careful selection of blood collection times based on clinical conditions, the choice of blood collection tubes based on ctDNA processing time and detection method, and the long-term storage of plasma samples at −80 °C to reduce repeated freeze-thaw cycles and minimize temperature changes.

Even with centrifugation and purification, there are still challenges in the process, such as the loss of a significant amount of DNA samples during purification and the potential contamination of tumor samples with DNA from blood cells during centrifugation (*Leest et al., 2020*; *Lehle et al., 2023*). To address these challenges, using suitable commercial kits, such as those from QIAamp, Microdiag®, and the MicroDiag® EGFR gene mutation detection kit, can enhance DNA purification and concentration (*Wang et al., 2021*).

Additionally, the Oxford Nanopore (ONT) platform provides a portable method for rapid genomic sequencing that can analyze DNA methylation without the need for complex sample processing, presenting new opportunities for real-time sequencing (*Katsman et al., 2022*).

Methods in cfDNA methylation analysis

The main methods of DNA methylation sequencing include whole-genome bisulfite sequencing (WGBS), reduced-representation bisulfite sequencing (RRBS), and methylated DNA immunoprecipitation sequencing (MeDIP-seq). WGBS is a technique commonly used to determine DNA methylation levels in the genome (*Jammula et al., 2020*; *Rajamäki et al., 2021*; *Tanaka et al., 2021*). It relies on two sequencing strategies: library construction and sequencing data, with various sequencing technologies and data analysis used to analyze the sequencing data. The most commonly used whole-genome sequencing technologies for WGBS include Illumina, PacBio, and Oxford Nanopore Technologies (*Gouil & Keniry, 2019*; *Alfaro et al., 2021*; *Zee et al., 2022*). RRBS is a newer-generation sequencing method with several advantages over WGBS. It uses approximately 5% of the genomic loci for sequencing analysis (*Liu et al., 2020b*; *Gu et al., 2021*; *Sharma et al., 2022*), resulting in reduced sequencing depth and coverage requirements, lower sequencing costs, and shorter sequencing times. RRBS also has high selectivity and accuracy, enabling the detection of DNA methylation status at single-base resolution in CpG sites. MeDIP-seq is a high-throughput sequencing-based epigenomic technology that immunoprecipitates methylated DNA fragments using specific antibodies before sequencing analysis (*Shen et al., 2019*; *Zhang et al., 2022*). This method provides high sequencing depth and coverage, enabling comprehensive analysis of DNA methylation across the genome, including CpG islands and non-CpG island regions. MeDIP-seq is particularly useful for identifying DNA methylation biomarkers and exploring the relationship between DNA methylation and gene regulation (*Zhou et al., 2018*; *Beck, Ben Maamar & Skinner, 2022*). However, these methods generate a large amount of information, leading to numerous differentially methylated regions (DMRs). However, they may have insufficient coverage and sequencing depth in certain regions, resulting in missing or false-positive results (*Ben Maamar et al., 2021*; *Gong et al., 2022*). Therefore, further validation of the selected DMRs is necessary. Targeted region methylation resequencing (Hi-MethylSeq), also known as bisulfite amplicon sequencing (BSAS), can accurately quantify methylation levels in multiple regions and loci of candidate genes in large populations based on WGBS, RRBS, and other studies. Thus, the Hi-MethylSeq technology serves as a powerful tool for subsequent validation of WGBS (*Cai et al., 2021*).

With the rapid development of sequencing technologies, there has been a continual improvement in sequencing quality. However, certain limitations still persist, such as the inability to directly identify the methylation states and sites of genes, which presents many challenges when analyzing gene expression, DNA modifications, and related aspects. To address these limitations, researchers have developed a methylation-sensitive restriction enzyme-based sequencing (MRE-seq) technology focused on gene enrichment. This technology enhances the size and concentration of DNA fragments by combining

methylation-sensitive restriction enzymes with high-throughput sequencing technology. Ultimately, it enables the comparison of the methylation states between different samples. For example, MRE-seq technology enables the sequencing of methylation sites in the DNA of both cancer and normal cells, allowing for a comparison of methylation status in different states and contributing to the study of the mechanism underlying cancer development (*Shin et al., 2022*). However, MRE-seq technology is limited to sequences containing CG sites and cannot directly detect other methylation sites, such as m6A. Therefore, it is necessary to employ complementary detection technologies to investigate the methylation status at these sites (*Sun, Wu & Ming, 2019*). Compared to sequencing methods that require specific enzyme cleavage and chemical reactions to determine DNA methylation status, the newly developed single-molecule real-time sequencing (SMRT) technology by the US-based PacBio company can directly achieve high-precision, high-throughput detection of DNA methylation status. This is achieved by monitoring the single-molecule long-chain amplification process of DNA polymerase on DNA templates while retaining the original DNA methylation status (*Forde et al., 2019*; *Chen et al., 2022*). In addition, nanopore sequencing technology reads DNA sequence information by measuring the transient electrical current changes in single DNA molecules passing through a nanopore (*Liu et al., 2021b*). This technology has a higher resolution and accuracy, can analyze longer DNA fragments for tasks such as genome-wide methylation spectrum analysis, and has a higher sensitivity for detecting low-frequency methylation sites (*Tourancheau et al., 2021*; *Katsman et al., 2022*). Recently, scholars such as *Yu et al. (2023)* compared the advantages and disadvantages of SMRT technology and nanopore sequencing technology across different long-read sequencing platforms. SMRT technology, based on a specialized chip for SMRT cells, limits the number of single reaction layers by restricting the position of the fixed DNA polymerase reaction. This restriction, in turn, limits the throughput and read length but generates data with a higher percentage of long cfDNA. In contrast, nanopores use a single-molecule electrical transfer chip with a nanopore, making the throughput and read length unlimited. Finally, we summarize recent clinical studies on the application of common sequencing techniques for cfDNA methylation in cancer research (*Bruzek et al., 2020*; *Zhang et al., 2020*; *Berchuck et al., 2022*; *Choy et al., 2022*; *Marinelli et al., 2022*) (Table 1).

Clinical application of cfDNA methylation in early detection of EC

Given the practicality of early cancer screening, analyzing cfDNA using simple and highly specific blood sampling may be more advantageous than traditional screening tools. It enables the analysis of tumors that are undetectable or uncertain using imaging techniques. Current research on cfDNA as a cancer biomarker has primarily focused on mutation detection, atypical fragment patterns, and abnormal methylation (*Zviran et al., 2020*; *Liu et al., 2021a*; *Esfahani et al., 2022*). In a CCGA study, *Jamshidi et al. (2022)* compared ten machine learning classifiers using various cfDNA features and found that cfDNA methylation patterns were the most promising for the early detection of various cancers. In this review, we focused on the research progress regarding cfDNA methylation-based biomarkers for early EC detection (Fig. 1).
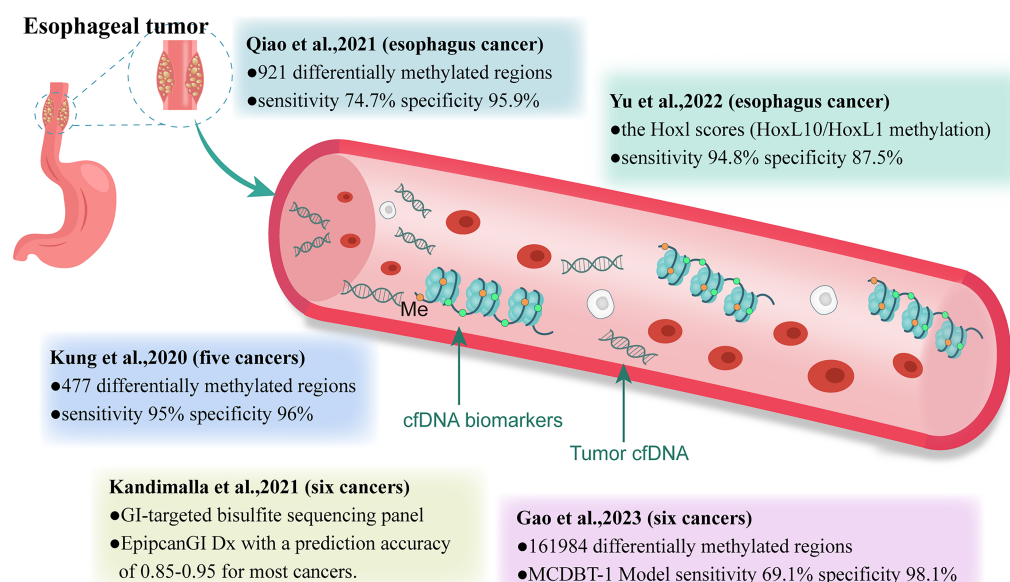
Table 1 Clinical applications of common methylation sequencing methods in tumors.

Author	Title	Sequencing method	Subject	Findings
Zhang et al. (2020)	Hypomethylation in HBV integration regions aids non-invasive surveillance to hepatocellular carcinoma by low-pass genome-wide bisulfite sequencing	WGBS	The methylation of hepatitis B virus integration regions and genome distribution of cfDNA	1. Methylation levels of integration sites certain candidate regions exhibited an area under the AUC value >0.85 to discriminate HCC from non-HCC samples; 2. The validation cohort achieved the prediction performance with an AUC of 0.954.
Marinelli et al. (2022)	Methylated DNA markers for plasma detection of ovarian cancer: discovery, validation, and clinical feasibility	RRBS	11-MDM panel highly discriminated OC from controls	96% (95% CI (89–99%)) specificity; 79% (69–87%) sensitivity, and the AUC 0.91 (0.86–0.96).
Berchuck et al. (2022)	Detecting neuroendocrine prostate cancer through tissue-informed cell-free DNA methylation analysis	MeDIP-seq	NEPC Risk Score	Applying the predefined NEPC Risk Score cutoff to the validation cohort resulted in 100% sensitivity and 95% specificity for detecting NEPC.
Choy et al. (2022)	Single-molecule sequencing enables long cell-free DNA detection and direct methylation analysis for cancer patients	Single-molecule real-time sequencing	HCC methylation score	The use of long cfDNA molecules demonstrated greatly discriminatory power (AUC: 0.91)
Bruzek et al. (2020)	Electronic DNA analysis of CSF cell-free tumor DNA to quantify multi-gene molecular response in pediatric high-grade glioma	Oxford Nanopore Technology	CSF cf-tDNA variant allele fraction	Nanopore demonstrated 85% sensitivity and 100% specificity in CSF samples with 0.1 femtomole DNA limit of detection and 12-hour results.

Notes.

HBV, hepatitis B virus; HCC, hepatocellular carcinoma; cfDNA, cell-free DNA; AUC, receiver operation curve; MDM, methylated DNA marker; OC, ovarian cancer; NEPV, neuroendocrine prostate cancer; CSF, cerebrospinal fluid; cf-tDNA, cell-free tumor DNA; WGBS, whole genome bisulfite sequencing; RRBS, reduced representation bisulfite sequencing; MeDIP-seq, methylated DNA immunoprecipitation sequencing.

DNA methylation is an important epigenetic modification that affects gene expression, genomic stability, and development. It has been widely used to assess cancer occurrence, progression, and treatment response (*Wu & Zhang, 2014*). Cytosine methylation (5-methylcytosine, 5mC) is a well-recognized epigenetic modification that affects gene expression. Reconstruction of DNA 5mC has been widely used to study cancer occurrence, progression, and treatment response (*Hu et al., 2021*). With the discovery of DNA demethylase-DNA dioxygenase (TET), the oxidative form of cytosine methylation modification has gained considerable attention (*Ito et al., 2011*). 5-hydroxymethylcytosine (5hmC) is the most common oxidative form of methylcytosine. It serves as an intermediate actively involved in demethylation and a stable modification form in the genome (*Bachman et al., 2014*; *Klungland & Robertson, 2017*). Many studies have confirmed that DNA modifications, such as 5mC and 5hmC, can be used as ideal biomarkers for cancer diagnosis (*Xiao et al., 2021*; *Zhang et al., 2021*; *Sjöström et al., 2022*; *Turpin & Salbert, 2022*). However, our study focused on the epigenetic modification of DNA methylation.

Esophageal tumor

Qiao et al.,2021 (esophagus cancer)
● 921 differentially methylated regions
● sensitivity 74.7% specificity 95.9%

Yu et al.,2022 (esophagus cancer)
● the Hoxl scores (HoxL10/HoxL1 methylation)
● sensitivity 94.8% specificity 87.5%

Kung et al.,2020 (five cancers)
● 477 differentially methylated regions
● sensitivity 95% specificity 96%

cfDNA biomarkers

Tumor cfDNA

Me

Kandimalla et al.,2021 (six cancers)
● GI-targeted bisulfite sequencing panel
● EpipcanGI Dx with a prediction accuracy
  of 0.85-0.95 for most cancers.

Gao et al.,2023 (six cancers)
● 161984 differentially methylated regions
● MCDBT-1 Model sensitivity 69.1% specificity 98.1%

Figure 1 **Clinical study of methylation-based cfDNA detection in cancer early screening.** GI, gastrointestinal; Me, methylation.

Full-size 🖼 DOI: 10.7717/peerj.16802/fig-1

The advantages of DNA methylation analysis over gene mutation analysis in cancer detection are as follows (Fig. 2): First, it has higher clinical sensitivity and a broader dynamic range, as there are many methylation targets in tumors and multiple altered CpG sites in each targeted genomic region. However, only a fraction of mutations in cancer tissue can be detected in circulating cfDNA (*Garcia et al., 2019*). Second, compared to the highly individualized and heterogeneous nature of gene mutations, which do not provide accurate tumor origin or specific organ information (*Shahbandi, Nguyen & Jackson, 2020*; *Maxwell et al., 2022*), different tissue-derived cfDNA presents distinct methylation patterns, enabling tissue tracing (*Baylin & Jones, 2011*). Finally, the number of methylation sites is significantly larger than the number of point mutations. Analyzing a cluster of CpG sites known as a methylation block (MB) as a complete unit can result in stronger methylation signals through both lateral patterns and longitudinal abundance (*Guo et al., 2017*).

Boldrin et al. (2020) conducted a study that analyzed the methylation status of long interspersed nucleotide element (LINE-1) sequences in 21 circulating cfDNA, 19 esophageal adenocarcinoma (EADC), and two Barrett's esophagus samples. They also performed a longitudinal analysis of two patients with Barrett's esophagus and one patient with EADC. This study revealed low levels of methylation of LINE-1 sequences in EADC cfDNA. Additionally, the longitudinal analysis indicated a correlation between the methylation status of LINE-1 sequences in cfDNA and the progression to EADC (*Boldrin et al., 2020*).

Several other studies have investigated the role of DNA methylation in the development and early detection of EC (*Wang et al., 2020*). *Fan et al. (2022)* confirmed that the frequency of P16 methylation increases with the severity of esophageal lesions and that it can serve
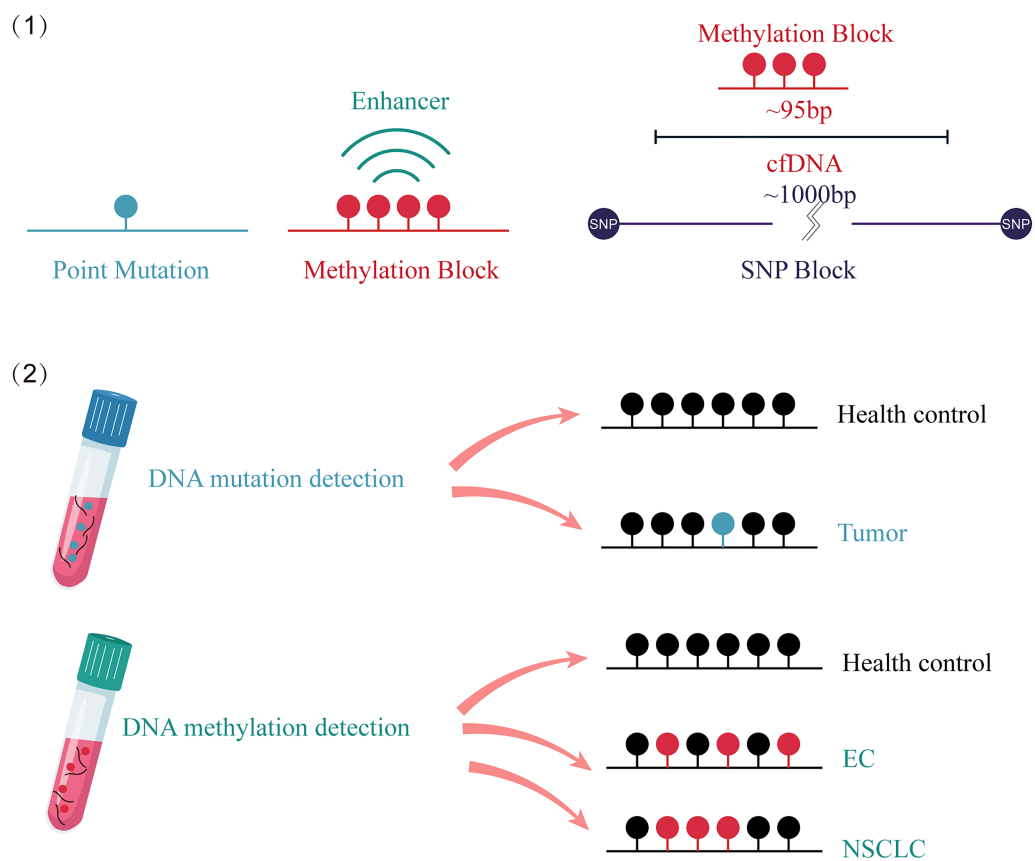
Figure 2 Comparison of the advantages between cfDNA methylation detection and cfDNA mutation detection. (1) Compared to point mutations, clustered and amplified signals are observed in cfDNA methylated sites. (2) cfDNA mutation detection can distinguish between cancer patients and healthy individuals. cfDNA methylation detection not only identifies cancer patients, but also traces the origin of the cancer. EC, esophagus cancer; NSCLC, non-small-cell lung cancer; SNP, single nucleotide polymorphism; cfDNA, cell-free DNA.

Full-size 🖼 DOI: 10.7717/peerj.16802/fig-2

as a biomarker for the early detection of ESCC and its precursor lesions. Scholars from Thailand have also found that P16 and TP53 methylation can serve as potential biomarkers for predicting EC, especially in individuals with drinking and smoking habits that can promote abnormal DNA methylation (*Poosari et al., 2022*). *Nasrollahzadeh et al. (2021)* compared the consistency of TP53 mutations in tumor tissues and cfDNA extracted from serum archives (the main source of cfDNA in retrospective studies) of 42 cases from high-risk areas in Iran and 39 matched controls. The results demonstrated a consistency of 24–36% between the variants detected in cfDNA from archived serum and paired FFPE ESCC tumor tissue, emphasizing the feasibility of early EC diagnosis through cfDNA detection (*Nasrollahzadeh et al., 2021*).

Qiao et al. (2021) developed an early screening model for EC based on cfDNA methylation. In their study, 161,984 cancer-related CpG sites were obtained from publicly available data in the TCGA and GEO databases, as well as internal data. Subsequently, 921 esophageal cancer-specific differentially methylated regions (DMRs) were identified and

optimized from esophageal tumors and their paired adjacent tissues and used to build an early screening model. This model was developed and tested on training and testing sets using plasma samples. Analyzing the model's performance on the training and testing sets showed a specificity of 95.2% for healthy individuals, 80% for benign esophageal diseases, and sensitivities ranging from 60%, 77.8%, 86.4%, to 100.0% for patients with EC at stages 0–IV, respectively (*Qiao et al., 2021*). These results preliminarily demonstrate the feasibility of liquid biopsy methylation detection for early screening of EC. To further validate the model's performance, *Qiao et al. (2021)* conducted a prospective, single-blind trial. In the independent validation group, the sensitivities for early (stages 0–II) and advanced (stages III–IV) esophageal cancers were 58.8% and 100.0%, respectively. Therefore, for patients highly suspected of having EC, it is worth considering whether a cfDNA methylation-based early screening model could replace commonly used auxiliary examinations as the primary diagnostic tool.

Yu et al. (2022) used whole-genome bisulfite sequencing (WGBS) to detect methylation features in ESCC and paired normal samples. Receiver operating characteristic curve analysis indicated that HOXC10 and HOXD1 methylation values were the best predictors for distinguishing ESCC samples from normal samples, achieving an AUC of 0.85. Consequently, the researchers developed an ESCC classification model based on a logit model using HOXC10/HOXD1 methylation status scores, which were validated by Sanger sequencing. The HOXL score effectively identified ESCC from normal samples, with an AUC of 0.96 (95% CI [0.91–0.99]) using an optimal threshold of 0.72, with a sensitivity of 94.8% and specificity of 87.5% (*Yu et al., 2022*). Later, *Qiao et al. (2021)*, analyzed 13 cfDNA samples obtained from the plasma of patients with ESCC using Sanger sequencing for HOXD1 and HOXC10 methylation. The results showed that HOXD1 CpG sites had a methylation rate of 90.77% (118/130), whereas HOXC10 CpG sites had a methylation rate of 60.67% (37/61) (*Yu et al., 2022*). These findings suggest that highly methylated HOXL paralogs, particularly the combination of HOXC10/HOXD1 methylation, have significant potential for the early detection of ESCC.

Researchers have developed early screening models for various cancers, including EC (*Liu et al., 2020a*; *Kandimalla et al., 2021*; *Gao et al., 2023*). *Chen et al. (2020)* from the University of California, USA, described a cancer screening test called PanSeer (based on noninvasive blood tests for circulating ctDNA methylation), which can detect cancer-specific methylation markers in the blood. During the study, the researchers reported preliminary results of PanSeer using plasma samples from 605 asymptomatic individuals, among whom 191 were subsequently diagnosed with gastric, esophageal, or liver cancer. The preliminary results of this study indicated that PanSeer detected five common types of cancer (gastric, esophageal, colorectal, lung, and liver cancers) in 88% (95% CI [80–93%]) of diagnosed patients, with a specificity of 96% (95% CI [93–98%]) and 95% (95% CI [89–98%]) of asymptomatic individuals who were later diagnosed with cancer (*Chen et al., 2020*). The significance of this study lies in PanSeer's ability to identify patients who have already developed cancer but are currently asymptomatic, rather than predicting patients who will develop cancer in the future. However, large-scale longitudinal clinical studies

are necessary to confirm the potential of cfDNA methylation for achieving early cancer detection before routine diagnosis.

Kandimalla et al. (2021) conducted a study where they obtained whole-genome 450k DNA methylation data for six gastrointestinal (GI) cancers and adjacent normal tissues from TCGA and GEO (GSE72872). They constructed a GI-targeted bisulfite sequencing panel (gitBS) using differentially methylated regions (DMRs) identified through analysis. This allowed them to retrospectively analyze the cfDNA methylation status of 300 patients with GI cancer and healthy individuals. They constructed and validated three panels for cancer detection using machine-learning algorithms. The panel was optimized to determine the minimum number of DMRs required for optimal detection performance (*Kandimalla et al., 2021*). The strengths of this research include achieving an AUC value of 0.94 for ESCC and 0.90 for EDAC in the GI single cancer detection panel, an AUC value of 0.88 in the GI pan-cancer detection panel, and an accuracy of 0.85–0.95 in the multi-GI cancer trace prediction panel (EpiPanGI Dx) (*Kandimalla et al., 2021*). Additionally, this model requires fewer biomarkers than previous studies (*Klein et al., 2021*), making it cost-effective and suitable for the development of diagnostic panels for large-scale clinical applications. In this study, the first 50 DMRs were sufficient to achieve optimal accuracy in GI single-cancer detection, while the first 150 information-rich DMRs achieved optimal performance in GI pan-cancer and multi-GI cancer classification models (*Kandimalla et al., 2021*).

Gao et al. (2023) constructed an early screening model for six cancers, including rectal, esophageal, liver, lung, ovarian, and pancreatic cancers. They built and validated a custom panel of 161,984 CpG sites using public and internal methylation databases. Subsequently, they retrospectively collected cfDNA samples from 1,693 participants to train and validate two different multi-cancer detection blood test models (MCDBT-1/2) under different clinical conditions. Both MCDBT-1 and MCDBT-2 models performed similarly, with MCDBT-1 having a screening specificity of up to 98.9% and a sensitivity of 69.1% in the independent validation cohort. The accuracy of cancer tissue tracing was 82.3%, and the real-world screening sensitivity of the MCDBT-1 model was 70.5%. Widespread adoption of this screening model could reduce the number of late-stage patients with these six common cancers by 38.7–46.4% and increase the relative five-year survival rate by 40%. In other words, implementing comprehensive early cancer screening based on this model could diagnose 38.7–46.4% of patients in the relatively early stages, addressing the current situation where approximately 60% of patients are diagnosed in the later stages. The absolute five-year survival rate of these six cancers could increase from 31.4% to 41.8–44.1% with curative treatments (*Gao et al., 2023*).

Overall, the application of cfDNA methylation in early tumor detection mainly focuses on two aspects: the high methylation of tumor suppressor gene promoter sites and the low methylation of oncogenes. For example, the tumor suppressor genes NRN1, JAM3, and RASSR2 are highly methylated in their promoter regions (*Guo et al., 2016*; *Du et al., 2021*; *Yang et al., 2022*), whereas PAX9, SIM2, and THSD4 are expressed in normal esophageal tissues but are downregulated in tumors (*Talukdar et al., 2021*). Nevertheless, it is crucial to note that the majority of participants in early screening models for EC have already been

diagnosed, which may introduce variability in the sensitivity for undiagnosed cases, posing difficulties in applying these research findings to the real world.

Based on cfDNA Methylation ongoing trials

In January 2023, based on the remarkable performance of cfDNA methylation in early cancer diagnosis, the "OverC™ Multi-Cancer Detection Blood Test" developed by Burning Rock Biotech (Irvine, CA, USA) on the ELSA-seq technology platform, was granted the Breakthrough Device Designation by the US FDA, becoming the third globally recognized multi-cancer early detection product with this designation. In May of the same year, Burning Rock Biotech published the latest results from the THUNDER study (NCT04820868) (*Gao et al., 2023*), which revealed the following key findings: (1) The MCDBT-1 model displayed leading international performance in six major types of cancer, including lung, liver, colorectal, ovarian, esophageal, and pancreatic cancers, with a specificity of 98.9% and a sensitivity of 69.1%; (2) The MCDBT-1 model demonstrated superior capabilities in predicting tissue origin, achieving prediction accuracies of 83.2% for TPO1 (primary origin) and 91.7% for TPO2 (primary and secondary origins); (3) In a real-world simulation, the MCDBT-1 model enabled the early diagnosis of 38.7%–46.4% of late-stage cancers (from stages III–IV to I–II), leading to a relative increase in the 5-year survival rate of these six cancer types by 33.1%–40.4%.

Currently, Burning Rock Biotech is collaborating with several clinical research centers on multiple large-scale research cohorts, including (1) PREDICT, the first prospective, multi-cancer early detection clinical trial in China with a cohort size of over ten thousand individuals; (2) PRESCIENT, the first prospective pan-cancer early detection study involving liquid biopsy and multi-omics analysis with a cohort size of ten thousand individuals; and (3) PREVENT, the first prospective, interventional early detection study targeting asymptomatic individuals with a cohort size of ten thousand individuals. The progress of these studies will accelerate the clinical validation of cfDNA methylation for early cancer diagnosis, addressing the need for various cancer screening methods, including esophageal cancer.

FUTURE PROSPECTS

Detecting cancer signals during the early stages of EC, when symptoms are not yet prominent and the disease has not yet progressed to a late stage, can potentially improve the success of surgical treatment. Recent research has shown significantly elevated cfDNA methylation levels in patients with EC compared to healthy individuals (*Salta et al., 2020*; *Talukdar et al., 2021*), and specific biomarkers unique to EC have been identified (*Li et al., 2019*; *Jammula et al., 2020*). Presently, an early cfDNA methylation screening model has been developed, with a sensitivity of up to 74.7% and a specificity of up to 95.9% (*Qiao et al., 2021*). Machine learning techniques combined with cfDNA methylation sequencing offer a useful approach for early cancer diagnosis. *Zhou et al. (2022)* utilized this approach to decode tumor information and determine the origin of the tumor, achieving a sensitivity of 86.1% and specificity of 94.7% for early cancer detection. Therefore, with the continuous development and improvement of cfDNA methylation sequencing technology, the cfDNA

early screening model we aim to establish can potentially detect asymptomatic cancer using regular blood tests before the diagnosis is confirmed.

While ctDNA methylation analysis has the potential to significantly improve early cancer screening methods and reduce cancer-related mortality, more research is needed to identify the most accurate cfDNA methylation markers suitable for large patient populations and the potential benefits of combining these markers with ctDNA mutation detection. In addition, large-scale clinical studies are necessary to assess the benefits of cfDNA methylation-based early screening models and the impact of early detection on patient survival.

In summary, the development and improvement of cfDNA methylation sequencing technology hold great promise for early detection of cancer in routine clinical settings. These advancements in early screening techniques have the potential to save lives and improve cancer treatment outcomes. Nevertheless, further research is needed to fully understand their impact and potential.

CONCLUSION

This review focuses on the recent advancements in early detection of esophageal cancer using cfDNA. Although standardized diagnostic methods may not always effectively detect the early stages of the disease, methylation-based detection has shown promise. Non-invasive liquid biopsy approaches significantly simplify the sample collection process, making diagnostic results easier to obtain and generally more reliable. This manuscript includes the latest research designs and their corresponding trial data, commonly using DNA methylation sequencing techniques to demonstrate the utility and effectiveness of cfDNA methylation in the early detection of esophageal cancer. With further clinical trials confirming the advantages of cfDNA methylation in early cancer diagnosis, cfDNA methylation could be incorporated into preventive care, resulting in substantial improvements in early detection of esophageal cancer at a low cost and with increased safety.

ADDITIONAL INFORMATION AND DECLARATIONS

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Rui Wang conceived and designed the experiments, analyzed the data, prepared figures and/or tables, and approved the final draft.
- Yue Yang performed the experiments, analyzed the data, authored or reviewed drafts of the article, and approved the final draft.
- Tianyu Lu conceived and designed the experiments, prepared figures and/or tables, and approved the final draft.
- Youbin Cui performed the experiments, prepared figures and/or tables, and approved the final draft.
- Bo Li conceived and designed the experiments, analyzed the data, authored or reviewed drafts of the article, and approved the final draft.
- Xin Liu conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.

Data Availability

The following information was supplied regarding data availability:

This is a literature review.

REFERENCES

Abbosh C, Birkbak NJ, Swanton C. 2018. Early stage NSCLC—challenges to implementing ctDNA-based screening and MRD detection. *Nature Reviews. Clinical Oncology* **15**:577–586 DOI 10.1038/s41571-018-0058-3.

Abbosh C, Birkbak NJ, Wilson GA, Jamal-Hanjani M, Constantin T, Salari R, Le Quesne J, Moore DA, Veeriah S, Rosenthal R, Marafioti T, Kirkizlar E, Watkins TBK, McGranahan N, Ward S, Martinson L, Riley J, Fraioli F, Al Bakir M, Grönroos E, Zambrana F, Endozo R, Bi WL, Fennessy FM, Sponer N, Johnson D, Laycock J, Shafi S, Czyzewska-Khan J, Rowan A, Chambers T, Matthews N, Turajlic S, Hiley C, Lee SM, Forster MD, Ahmad T, Falzon M, Borg E, Lawrence D, Hayward M, Kolvekar S, Panagiotopoulos N, Janes SM, Thakrar R, Ahmed A, Blackhall F, Summers Y, Hafez D, Naik A, Ganguly A, Kareht S, Shah R, Joseph L, Marie Quinn A, Crosbie PA, Naidu B, Middleton G, Langman G, Trotter S, Nicolson M, Remmen H, Kerr K, Chetty M, Gomersall L, Fennell DA, Nakas A, Rathinam S, Anand G, Khan S, Russell P, Ezhil V, Ismail B, Irvin-Sellers M, Prakash V, Lester JF, Kornaszewska M, Attanoos R, Adams H, Davies H, Oukrif D, Akarca AU, Hartley JA, Lowe HL, Lock S, Iles N, Bell H, Ngai Y, Elgar G, Szallasi Z, Schwarz RF, Herrero J, Stewart A, Quezada SA, Peggs KS, Van Loo P, Dive C, Lin CJ, Rabinowitz M, Aerts HJWL, Hackshaw A, Shaw JA, Zimmermann BG, TRACERx consortium, PEACE consortium, Swanton C. 2017. Phylogenetic

ctDNA analysis depicts early-stage lung cancer evolution. *Nature* **545**:446–451 DOI 10.1038/nature22364.

Alfaro JA, Bohländer P, Dai M, Filius M, Howard CJ, van Kooten XF, Ohayon S, Pomorski A, Schmid S, Aksimentiev A, Anslyn EV, Bedran G, Cao C, Chinappi M, Coyaud E, Dekker C, Dittmar G, Drachman N, Eelkema R, Goodlett D, Hentz S, Kalathiya U, Kelleher NL, Kelly RT, Kelman Z, Kim SH, Kuster B, Rodriguez-Larrea D, Lindsay S, Maglia G, Marcotte EM, Marino JP, Masselon C, Mayer M, Samaras P, Sarthak K, Sepiashvili L, Stein D, Wanunu M, Wilhelm M, Yin P, Meller A, Joo C. 2021. The emerging landscape of single-molecule protein sequencing technologies. *Nature Methods* **18**:604–617 DOI 10.1038/s41592-021-01143-1.

Azad TD, Chaudhuri AA, Fang P, Qiao Y, Esfahani MS, Chabon JJ, Hamilton EG, Yang YD, Lovejoy A, Newman AM, Kurtz DM, Jin M, Schroers-Martin J, Stehr H, Liu CL, Hui AB, Patel V, Maru D, Lin SH, Alizadeh AA, Diehn M. 2020. Circulating tumor DNA analysis for detection of minimal residual disease after chemoradiotherapy for localized esophageal cancer. *Gastroenterology* **158**:494–505 DOI 10.1053/j.gastro.2019.10.039.

Bachman M, Uribe-Lewis S, Yang X, Williams M, Murrell A, Balasubramanian S. 2014. 5-hydroxymethylcytosine is a predominantly stable DNA modification. *Nature Chemistry* **6**:1049–1055 DOI 10.1038/nchem.2064.

Baylin SB, Jones PA. 2011. A decade of exploring the cancer epigenome—biological and translational implications. *Nature Reviews. Cancer* **11**:726–734 DOI 10.1038/nrc3130.

Beck D, Ben Maamar M, Skinner MK. 2022. Genome-wide CpG density and DNA methylation analysis method (MeDIP, RRBS, and WGBS) comparisons. *Epigenetics* **17**:518–530 DOI 10.1080/15592294.2021.1924970.

Ben Maamar M, Sadler-Riggleman I, Beck D, Skinner MK. 2021. Genome-wide mapping of DNA methylation 5mC by methylated DNA immunoprecipitation (MeDIP)-sequencing. *Methods in Molecular Biology* **2198**:301–310 DOI 10.1007/978-1-0716-0876-0_23.

Berchuck JE, Baca SC, McClure HM, Korthauer K, Tsai HK, Nuzzo PV, Kelleher KM, He M, Steinharter JA, Zacharia S, Spisak S, Seo JH, Conteduca V, Elemento O, Auh J, Sigouros M, Corey E, Hirsch MS, Taplin ME, Choueiri TK, Pomerantz MM, Beltran H, Freedman ML. 2022. Detecting neuroendocrine prostate cancer through tissue-informed cell-free DNA methylation analysis. *Clinical Cancer Research* **28**:928–938 DOI 10.1158/1078-0432.CCR-21-3762.

Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, Bartlett BR, Wang H, Luber B, Alani RM, Antonarakis ES, Azad NS, Bardelli A, Brem H, Cameron JL, Lee CC, Fecher LA, Gallia GL, Gibbs P, Le D, Giuntoli RL, Goggins M, Hogarty MD, Holdhoff M, Hong SM, Jiao Y, Juhl HH, Kim JJ, Siravegna G, Laheru DA, Lauricella C, Lim M, Lipson EJ, Marie SK, Netto GJ, Oliner KS, Olivi A, Olsson L, Riggins GJ, Sartore-Bianchi A, Schmidt K, Shih lM, Oba-Shinjo SM, Siena S, Theodorescu D, Tie J, Harkins TT, Veronese S, Wang TL, Weingart JD, Wolfgang CL, Wood LD, Xing D, Hruban RH, Wu J, Allen PJ, Schmidt CM, Choti MA, Velculescu VE, Kinzler KW, Vogelstein B, Papadopoulos N, Diaz Jr LA. 2014.

Detection of circulating tumor DNA in early- and late-stage human malignancies. *Science Translational Medicine* **6**:224ra24 DOI 10.1126/scitranslmed.3007094.

Birknerova N, Mancikova V, Paul ED, Matyasovsky J, Cekan P, Palicka V, Parova H. 2022. Circulating cell-free DNA-based methylation pattern in saliva for early diagnosis of head and neck cancer. *Cancer* **14(19)** DOI 10.3390/cancers14194882.

Boldrin E, Curtarello M, Dallan M, Alfieri R, Realdon S, Fassan M, Saggioro D. 2020. Detection of LINE-1 hypomethylation in cfDNA of Esophageal adenocarcinoma Patients. *International Journal of Molecular Sciences* **21(4)** DOI 10.3390/ijms21041547.

Bruzek AK, Ravi K, Muruganand A, Wadden J, Babila CM, Cantor E, Tunkle L, Wierzbicki K, Stallard S, Dickson RP, Wolfe I, Mody R, Schwartz J, Franson A, Robertson PL, Muraszko KM, Maher CO, Garton HJL, Qin T, Koschmann C. 2020. Electronic DNA analysis of CSF cell-free tumor DNA to quantify multi-gene molecular response in pediatric high-grade glioma. *Clinical Cancer Research* **26**:6266–6276 DOI 10.1158/1078-0432.CCR-20-2066.

Cai T, Qin Q, Song R, Zhao J, Wang G, Zhang J. 2021. Identifying and validating differentially methylated regions in newly diagnosed patients with Graves' disease. *DNA and Cell Biology* **40**:482–490 DOI 10.1089/dna.2020.6215.

Chen J, Cheng J, Chen X, Inoue M, Liu Y, Song CX. 2022. Whole-genome long-read TAPS deciphers DNA methylation patterns at base resolution using PacBio SMRT sequencing technology. *Nucleic Acids Research* **50**:e104 DOI 10.1093/nar/gkac612.

Chen X, Gole J, Gore A, He Q, Lu M, Min J, Yuan Z, Yang X, Jiang Y, Zhang T, Suo C, Li X, Cheng L, Zhang Z, Niu H, Li Z, Xie Z, Shi H, Zhang X, Fan M, Wang X, Yang Y, Dang J, McConnell C, Zhang J, Wang J, Yu S, Ye W, Gao Y, Zhang K, Liu R, Jin L. 2020. Non-invasive early detection of cancer four years before conventional diagnosis using a blood test. *Nature Communications* **11**:3475 DOI 10.1038/s41467-020-17316-z.

Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J. 2016. Cancer statistics in China, 2015. *CA: A Cancer Journal for Clinicians* **66**:115–132 DOI 10.3322/caac.21338.

Chen R, Zheng R, Zhang S, Wang S, Sun K, Zeng H, Li L, Wei W, He J. 2023. Patterns and trends in esophageal cancer incidence and mortality in China: an analysis based on cancer registry data. *Journal of the National Cancer Center* **3**:21–27 DOI 10.1016/j.jncc.2023.01.002.

Chiu RW, Akolekar R, Zheng YW, Leung TY, Sun H, Chan KC, Lun FM, Go AT, Lau ET, To WW, Leung WC, Tang RY, Au-Yeung SK, Lam H, Kung YY, Zhang X, van Vugt JM, Minekawa R, Tang MH, Wang J, Oudejans CB, Lau TK, Nicolaides KH, Lo YM. 2011. Non-invasive prenatal assessment of trisomy 21 by multiplexed maternal plasma DNA sequencing: large scale validity study. *BMJ* **342**:c7401 DOI 10.1136/bmj.c7401.

Choy LYL, Peng W, Jiang P, Cheng SH, Yu SCY, Shang H, Olivia Tse OY, Wong J, Wong VWS, Wong GLH, Lam WKJ, Chan SL, Chiu RWK, Chan KCA, Lo YMD. 2022. Single-molecule sequencing enables long cell-free DNA detection and

direct methylation analysis for cancer patients. *Clinical Chemistry* **68**:1151–1163 DOI 10.1093/clinchem/hvac086.

Cohen JD, Li L, Wang Y, Thoburn C, Afsari B, Danilova L, Douville C, Javed AA, Wong F, Mattox A, Hruban RH, Wolfgang CL, Goggins MG, Dal Molin M, Wang TL, Roden R, Klein AP, Ptak J, Dobbyn L, Schaefer J, Silliman N, Popoli M, Vogelstein JT, Browne JD, Schoen RE, Brand RE, Tie J, Gibbs P, Wong HL, Mansfield AS, Jen J, Hanash SM, Falconi M, Allen PJ, Zhou S, Bettegowda C, Diaz Jr LA, Tomasetti C, Kinzler KW, Vogelstein B, Lennon AM, Papadopoulos N. 2018. Detection and localization of surgically resectable cancers with a multi-analyte blood test. *Science* **359**:926–930 DOI 10.1126/science.aar3247.

Dasari A, Morris VK, Allegra CJ, Atreya C, Benson 3rd AB, Boland P, Chung K, Copur MS, Corcoran RB, Deming DA, Dwyer A, Diehn M, Eng C, George TJ, Gollub MJ, Goodwin RA, Hamilton SR, Hechtman JF, Hochster H, Hong TS, Innocenti F, Iqbal A, Jacobs SA, Kennecke HF, Lee JJ, Lieu CH, Lenz HJ, Lindwasser OW, Montagut C, Odisio B, Ou FS, Porter L, Raghav K, Schrag D, Scott AJ, Shi Q, Strickler JH, Venook A, Yaeger R, Yothers G, You YN, Zell JA, Kopetz S. 2020. ctDNA applications and integration in colorectal cancer: an NCI colon and rectal-anal task forces whitepaper. *Nature Reviews. Clinical Oncology* **17**:757–770 DOI 10.1038/s41571-020-0392-0.

Du W, Gao A, Herman JG, Wang L, Zhang L, Jiao S, Guo M. 2021. Methylation of NRN1 is a novel synthetic lethal marker of PI3K-Akt-mTOR and ATR inhibitors in esophageal cancer. *Cancer Science* **112**:2870–2883 DOI 10.1111/cas.14917.

Egyud M, Tejani M, Pennathur A, Luketich J, Sridhar P, Yamada E, Ståhlberg A, Filges S, Krzyzanowski P, Jackson J, Kalatskaya I, Jiao W, Nielsen G, Zhou Z, Litle V, Stein L, Godfrey T. 2019. Detection of circulating tumor DNA in plasma: a potential biomarker for esophageal adenocarcinoma. *Annals of Thoracic Surgery* **108**:343–349 DOI 10.1016/j.athoracsur.2019.04.004.

Esfahani MS, Hamilton EG, Mehrmohamadi M, Nabet BY, Alig SK, King DA, Steen CB, Macaulay CW, Schultz A, Nesselbush MC, Soo J, Schroers-Martin JG, Chen B, Binkley MS, Stehr H, Chabon JJ, Sworder BJ, Hui AB, Frank MJ, Moding EJ, Liu CL, Newman AM, Isbell JM, Rudin CM, Li BT, Kurtz DM, Diehn M, Alizadeh AA. 2022. Inferring gene expression from cell-free DNA fragmentation profiles. *Nature Biotechnology* **40**:585–597 DOI 10.1038/s41587-022-01222-4.

Fan Z, Qin Y, Zhou J, Chen R, Gu J, Li M, Zhou J, Li X, Lin D, Wang J, Deng D, Wei W. 2022. Feasibility of using P16 methylation as a cytologic marker for esophageal squamous cell carcinoma screening: a pilot study. *Cancer Medicine* **11**:4033–4042 DOI 10.1002/cam4.4718.

Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. 2010. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *International Journal of Cancer* **127**:2893–2917 DOI 10.1002/ijc.25516.

Forde BM, McAllister LJ, Paton JC, Paton AW, Beatson SA. 2019. SMRT sequencing reveals differential patterns of methylation in two O111:H- STEC isolates from

a hemolytic uremic syndrome outbreak in Australia. *Scientific Reports* **9**:9436 DOI 10.1038/s41598-019-45760-5.

Freitas C, Sousa C, Machado F, Serino M, Santos V, Cruz-Martins N, Teixeira A, Cunha A, Pereira T, Oliveira HP, Costa JL, Hespanhol V. 2021. The role of liquid biopsy in early diagnosis of lung cancer. *Frontiers in Oncology* **11**:634316 DOI 10.3389/fonc.2021.634316.

Gao Q, Lin YP, Li BS, Wang GQ, Dong LQ, Shen BY, Lou WH, Wu WC, Ge D, Zhu QL, Xu Y, Xu JM, Chang WJ, Lan P, Zhou PH, He MJ, Qiao GB, Chuai SK, Zang RY, Shi TY, Tan LJ, Yin J, Zeng Q, Su XF, Wang ZD, Zhao XQ, Nian WQ, Zhang S, Zhou J, Cai SL, Zhang ZH, Fan J. 2023. Unintrusive multi-cancer detection by circulating cell-free DNA methylation sequencing (THUNDER): development and independent validation studies. *Annals of Oncology* **34**:486–495 DOI 10.1016/j.annonc.2023.02.010.

Garcia J, Wozny AS, Geiguer F, Delherme A, Barthelemy D, Merle P, Tissot C, Jones FS, Johnson C, Xing X, Xu Z, Edelstein DL, Brevet M, Souquet PJ, Rodriguez-Lafrasse C, Payen L, Couraud S. 2019. Profiling of circulating tumor DNA in plasma of non-small cell lung cancer patients, monitoring of epidermal growth factor receptor p.T790M mutated allelic fraction using beads, emulsion, amplification, and magnetics companion assay and evaluation in future application in mimicking circulating tumor cells. *Cancer Medicine* **8**:3685–3697 DOI 10.1002/cam4.2244.

Gong T, Borgard H, Zhang Z, Chen S, Gao Z, Deng Y. 2022. Analysis and performance assessment of the whole genome bisulfite sequencing data workflow: currently available tools and a practical guide to advance DNA methylation studies. *Small Methods* **6**:e2101251 DOI 10.1002/smtd.202101251.

Gouil Q, Keniry A. 2019. Latest techniques to study DNA methylation. *Essays in Biochemistry* **63**:639–648 DOI 10.1042/EBC20190027.

Gu H, Raman AT, Wang X, Gaiti F, Chaligne R, Mohammad AW, Arczewska A, Smith ZD, Landau DA, Aryee MJ, Meissner A, Gnirke A. 2021. Smart-RRBS for single-cell methylome and transcriptome analysis. *Nature Protocols* **16**:4004–4030 DOI 10.1038/s41596-021-00571-9.

Guo S, Diep D, Plongthongkum N, Fung HL, 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. *Nature Genetics* **49**:635–642 DOI 10.1038/ng.3805.

Guo W, Dong Z, Cui J, Guo Y, Shen S, Guo X, Kuang G. 2016. Aberrant hypermethylation of RASSF2 in tumors and peripheral blood DNA as a biomarker for malignant progression and poor prognosis of esophageal squamous cell carcinoma. *Clinical and Experimental Metastasis* **33**:73–85 DOI 10.1007/s10585-015-9759-5.

Heitzer E, Auinger L, Speicher MR. 2020. Cell-free DNA and apoptosis: how dead cells inform about the living. *Trends in Molecular Medicine* **26**:519–528 DOI 10.1016/j.molmed.2020.01.012.

Herberts C, Annala M, Sipola J, Ng SWS, Chen XE, Nurminen A, Korhonen OV, Munzur AD, Beja K, Schönlau E, Bernales CQ, Ritch E, Bacon JVW, Lack NA,

Nykter M, Aggarwal R, Small EJ, Gleave ME, SU2C/PCF West Coast Prostate Cancer Dream Team, Quigley DA, Feng FY, Chi KN, Wyatt AW. 2022. Deep whole-genome ctDNA chronology of treatment-resistant prostate cancer. *Nature* **608**:199–208 DOI 10.1038/s41586-022-04975-9.

Hou QF, Wu D, Chu Y, Kang B, Liao SX, Yang YL, Zhang CY, Zhang JX, Wu G. 2012. Clinical application of noninvasive prenatal diagnosis using cell free fetal DNA in maternal plasma. *Zhonghua Fu Chan Ke Za Zhi* **47**:813–817.

Hu J, Othmane B, Yu A, Li H, Cai Z, Chen X, Ren W, Chen J, Zu X. 2021. 5-mC regulator-mediated molecular subtypes depict the hallmarks of the tumor microenvironment and guide precision medicine in bladder cancer. *BMC Medicine* **19**:289 DOI 10.1186/s12916-021-02163-6.

Ignatiadis M, Sledge GW, Jeffrey SS. 2021. Liquid biopsy enters the clinic— implementation issues and future challenges. *Nature Reviews. Clinical Oncology* **18**:297–312 DOI 10.1038/s41571-020-00457-x.

Ito S, Shen L, Dai Q, Wu SC, Collins LB, Swenberg JA, He C, Zhang Y. 2011. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science* **333**:1300–1303 DOI 10.1126/science.1210597.

Jammula S, Katz-Summercorn AC, Li X, Linossi C, Smyth E, Killcoyne S, Biasci D, Subash VV, Abbas S, Blasko A, Devonshire G, Grantham A, Wronowski F, O'Donovan M, Grehan N, Eldridge MD, Tavaré S, Fitzgerald RC. 2020. Identification of subtypes of Barrett's esophagus and esophageal adenocarcinoma based on DNA methylation profiles and integration of transcriptome and genome data. *Gastroenterology* **158**:1682–1697 DOI 10.1053/j.gastro.2020.01.044.

Jamshidi A, Liu MC, Klein EA, Venn O, Hubbell E, Beausang JF, Gross S, Melton C, Fields AP, Liu Q, Zhang N, Fung ET, Kurtzman KN, Amini H, Betts C, Civello D, Freese P, Calef R, Davydov K, Fayzullina S, Hou C, Jiang R, Jung B, Tang S, Demas V, Newman J, Sakarya O, Scott E, Shenoy A, Shojaee S, Steffen KK, Nicula V, Chien TC, Bagaria S, Hunkapiller N, Desai M, Dong Z, Richards DA, Yeatman TJ, Cohn AL, Thiel DD, Berry DA, Tummala MK, McIntyre K, Sekeres MA, Bryce A, Aravanis AM, Seiden MV, Swanton C. 2022. Evaluation of cell-free DNA approaches for multi-cancer early detection. *Cancer Cell* **40**:1537–1549 DOI 10.1016/j.ccell.2022.10.022.

Jen J, Wu L, Sidransky D. 2000. An overview on the isolation and analysis of circulating tumor DNA in plasma and serum. *Annals of the New York Academy of Sciences* **906**:8–12 DOI 10.1111/j.1749-6632.2000.tb06581.x.

Jiang M, Zhou J, Xie X, Huang Z, Liu R, Lv Y. 2022. Single nanoparticle counting-based liquid biopsy for cancer diagnosis. *Analytical Chemistry* **94**:15433–15439 DOI 10.1021/acs.analchem.2c03367.

Jin Y, Chen DL, Wang F, Yang CP, Chen XX, You JQ, Huang JS, Shao Y, Zhu DQ, Ouyang YM, Luo HY, Wang ZQ, Wang FH, Li YH, Xu RH, Zhang DS. 2020. The predicting role of circulating tumor DNA landscape in gastric cancer patients treated with immune checkpoint inhibitors. *Molecular Cancer* **19**:154 DOI 10.1186/s12943-020-01274-7.

Kandimalla R, Xu J, Link A, Matsuyama T, Yamamura K, Parker MI, Uetake H, Balaguer F, Borazanci E, Tsai S, Evans D, Meltzer SJ, Baba H, Brand R, Von Hoff D, Li W, Goel A. 2021. EpiPanGI Dx: a cell-free DNA methylation fingerprint for the early detection of gastrointestinal cancers. *Clinical Cancer Research* **27**:6135–6144 DOI 10.1158/1078-0432.CCR-21-1982.

Katsman E, Orlanski S, Martignano F, Fox-Fisher I, Shemer R, Dor Y, Zick A, Eden A, Petrini I, Conticello SG, Berman BP. 2022. Detecting cell-of-origin and cancer-specific methylation features of cell-free DNA from nanopore sequencing. *Genome Biology* **23**:158 DOI 10.1186/s13059-022-02710-1.

Kim AK, Hamilton JP, Lin SY, Chang TT, Hann HW, Hu CT, Lou Y, Lin YJ, Gade TP, Park G, Luu H, Lee TJ, Wang J, Chen D, Goggins MG, Jain S, Song W, Su YH. 2022. Urine DNA biomarkers for hepatocellular carcinoma screening. *British Journal of Cancer* **126**:1432–1438 DOI 10.1038/s41416-022-01706-9.

Klein EA, Richards D, Cohn A, Tummala M, Lapham R, Cosgrove D, Chung G, Clement J, Gao J, Hunkapiller N, Jamshidi A, Kurtzman KN, Seiden MV, Swanton C, Liu MC. 2021. Clinical validation of a targeted methylation-based multi-cancer early detection test using an independent validation set. *Annals of Oncology* **32**:1167–1177 DOI 10.1016/j.annonc.2021.05.806.

Klungland A, Robertson AB. 2017. Oxidized C5-methyl cytosine bases in DNA: 5-hydroxymethylcytosine; 5-formylcytosine; and 5-carboxycytosine. *Free Radical Biology and Medicine* **107**:62–68 DOI 10.1016/j.freeradbiomed.2016.11.038.

Leest PV, Boonstra PA, Elst AT, Kempen LCV, Tibbesma M, Koopmans J, Miedema A, Tamminga M, Groen HJM, Reyners AKL, Schuuring E. 2020. Comparison of circulating cell-free DNA extraction methods for downstream analysis in cancer patients. *Cancer* **12(5)** DOI 10.3390/cancers12051222.

Lehle S, Emons J, Hack CC, Heindl F, Hein A, Preuß C, Seitz K, Zahn AL, Beckmann MW, Fasching PA, Ruebner M, Huebner H. 2023. Evaluation of automated techniques for extraction of circulating cell-free DNA for implementation in standardized high-throughput workflows. *Scientific Reports* **13**:373 DOI 10.1038/s41598-022-27216-5.

Li N, Wang BX, Li J, Shao Y, Li MT, Li JJ, Kuang PP, Liu Z, Sun TY, Wu HQ, Ou W, Wang SY. 2022. Perioperative circulating tumor DNA as a potential prognostic marker for operable stage I to IIIA non-small cell lung cancer. *Cancer* **128**:708–718 DOI 10.1002/cncr.33985.

Li D, Zhang L, Liu Y, Sun H, Onwuka JU, Zhao Z, Tian W, Xu J, Zhao Y, Xu H. 2019. Specific DNA methylation markers in the diagnosis and prognosis of esophageal cancer. *Aging* **11**:11640–11658 DOI 10.18632/aging.102569.

Liang H, Fan JH, Qiao YL. 2017. Epidemiology, etiology, and prevention of esophageal squamous cell carcinoma in China. *Cancer Biology and Medicine* **14**:33–41 DOI 10.20892/j.issn.2095-3941.2016.0093.

Liu Y, Han Y, Zhou L, Pan X, Sun X, Liu Y, Liang M, Qin J, Lu Y, Liu P. 2020b. A comprehensive evaluation of computational tools to identify differential methylation regions using RRBS data. *Genomics* **112**:4567–4576 DOI 10.1016/j.ygeno.2020.07.032.

Liu MC, Oxnard GR, Klein EA, Swanton C, Seiden MV, Consortium CCGA. 2020a. Sensitive and specific multi-cancer detection and localization using methylation signatures in cell-free DNA. *Annals of Oncology* **31**:745–759 DOI 10.1016/j.annonc.2020.02.011.

Liu Y, Rosikiewicz W, Pan Z, Jillette N, Wang P, Taghbalout A, Foox J, Mason C, Carroll M, Cheng A, Li S. 2021b. DNA methylation-calling tools for Oxford nanopore sequencing: a survey and human epigenome-wide evaluation. *Genome Biology* **22**:295 DOI 10.1186/s13059-021-02510-z.

Lo YM, Chan KC, Sun H, Chen EZ, Jiang P, Lun FM, Zheng YW, Leung TY, Lau TK, Cantor CR, Chiu RW. 2010. Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus. *Science Translational Medicine* **2**:61ra91 DOI 10.1126/scitranslmed.3001720.

Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, Wainscoat JS. 1997. Presence of fetal DNA in maternal plasma and serum. *Lancet* **350**:485–487 DOI 10.1016/S0140-6736(97)02174-0.

Liu J, Zhao H, Huang Y, Xu S, Zhou Y, Zhang W, Li J, Ming Y, Wang X, Zhao S, Li K, Dong X, Ma Y, Qian T, Chen X, Xing Z, Zhang Y, Chen H, Liu Z, Pang D, Zhou M, Wu Z, Wang X, Wang X, Wu N, Su J. 2021a. Genome-wide cell-free DNA methylation analyses improve accuracy of non-invasive diagnostic imaging for early-stage breast cancer. *Molecular Cancer* **20**:36 DOI 10.1186/s12943-021-01330-w.

Lo YM, Zhang J, Leung TN, Lau TK, Chang AM, Hjelm NM. 1999. Rapid clearance of fetal DNA from maternal plasma. *American Journal of Human Genetics* **64**:218–224 DOI 10.1086/302205.

Loyfer N, Magenheim J, Peretz A, Cann G, Bredno J, Klochendler A, Fox-Fisher I, Shabi-Porat S, Hecht M, Pelet T, Moss J, Drawshy Z, Amini H, Moradi P, Nagaraju S, Bauman D, Shveiky D, Porat S, Dior U, Rivkin G, Or O, Hirshoren N, Carmon E, Pikarsky A, Khalaileh A, Zamir G, Grinbaum R, Gazala MAbu, Mizrahi I, Shussman N, Korach A, Wald O, Izhar U, Erez E, Yutkin V, Samet Y, Rotnemer Golinkin D, Spalding KL, Druid H, Arner P, Shapiro AMJ, Grompe M, Aravanis A, Venn O, Jamshidi A, Shemer R, Dor Y, Glaser B, Kaplan T. 2023. A DNA methylation atlas of normal human cell types. *Nature* **613**:355–364 DOI 10.1038/s41586-022-05580-6.

Luo H, Zhao Q, Wei W, Zheng L, Yi S, Li G, Wang W, Sheng H, Pu H, Mo H, Zuo Z, Liu Z, Li C, Xie C, Zeng Z, Li W, Hao X, Liu Y, Cao S, Liu W, Gibson S, Zhang K, Xu G, Xu RH. 2020. Circulating tumor DNA methylation profiles enable early diagnosis, prognosis prediction, and screening for colorectal cancer. *Science Translational Medicine* **12(524)** DOI 10.1126/scitranslmed.aax7533.

Marinelli LM, Kisiel JB, Slettedahl SW, Mahoney DW, Lemens MA, Shridhar V, Taylor WR, Staub JK, Cao X, Foote PH, Burger KN, Berger CK, O'Connell MC, Doering KA, Giakoumopoulos M, Berg H, Volkmann C, Solsrud A, Allawi HT, Kaiser M, Vaccaro AM, Crawford CAlbright, Moehlenkamp C, Shea G, Deist MS, Schoolmeester JK, Kerr SE, Sherman ME, Bakkum-Gamez JN. 2022. Methylated DNA markers for plasma detection of ovarian cancer: discovery, validation, and clinical feasibility. *Gynecologic Oncology* **165**:568–576 DOI 10.1016/j.ygyno.2022.03.018.

Maxwell KN, Cheng HH, Powers J, Gulati R, Ledet EM, Morrison C, Le A, Hausler R, Stopfer J, Hyman S, Kohlmann W, Naumer A, Vagher J, Greenberg SE, Naylor L, Laurino M, Konnick EQ, Shirts BH, AlDubayan SH, Van Allen EM, Nguyen B, Vijai J, Abida W, Carlo MI, Dubard-Gault M, Lee DJ, Maese LD, Mandelker D, Montgomery B, Morris MJ, Nicolosi P, Nussbaum RL, Schwartz LE, Stadler Z, Garber JE, Offit K, Schiffman JD, Nelson PS, Sartor O, Walsh MF, Pritchard CC. 2022. Inherited TP53 variants and risk of prostate cancer. *European Urology* **81**:243–250 DOI 10.1016/j.eururo.2021.10.036.

McEwen AE, Leary SES, Lockwood CM. 2020. Beyond the blood: CSF-derived cfDNA for diagnosis and characterization of CNS tumors. *Frontiers in Cell and Developmental Biology* **8**:45 DOI 10.3389/fcell.2020.00045.

Meddeb R, Dache ZAA, Thezenas S, Otandault A, Tanos R, Pastor B, Sanchez C, Azzi J, Tousch G, Azan S, Mollevi C, Adenis A, Messaoudi SEl, Blache P, Thierry AR. 2019. Quantifying circulating cell-free DNA in humans. *Scientific Reports* **9**:5220 DOI 10.1038/s41598-019-41593-4.

Morgan E, Soerjomataram I, Rumgay H, Coleman HG, Thrift AP, Vignat J, Laversanne M, Ferlay J, Arnold M. 2022. The global landscape of esophageal squamous cell carcinoma and esophageal adenocarcinoma incidence and mortality in 2020 and projections to 2040: new estimates from GLOBOCAN 2020. *Gastroenterology* **163**:649–658 DOI 10.1053/j.gastro.2022.05.054.

Nakamura Y, Okamoto W, Kato T, Esaki T, Kato K, Komatsu Y, Yuki S, Masuishi T, Nishina T, Ebi H, Sawada K, Taniguchi H, Fuse N, Nomura S, Fukui M, Matsuda S, Sakamoto Y, Uchigata H, Kitajima K, Kuramoto N, Asakawa T, Olsen S, Odegaard JI, Sato A, Fujii S, Ohtsu A, Yoshino T. 2021. Circulating tumor DNA-guided treatment with pertuzumab plus trastuzumab for HER2-amplified metastatic colorectal cancer: a phase 2 trial. *Nature Medicine* **27**:1899–1903 DOI 10.1038/s41591-021-01553-w.

Nasrollahzadeh D, Roshandel G, Delhomme TM, Avogbe PH, Foll M, Saidi F, Poustchi H, Sotoudeh M, Malekzadeh R, Brennan P, McKay J, Hainaut P, Abedi-Ardekani B. 2021. TP53 targeted deep sequencing of cell-free DNA in esophageal squamous cell carcinoma using low-quality serum: concordance with tumor mutation. *International Journal of Molecular Sciences* **22**(11) DOI 10.3390/ijms22115627.

Osumi H, Shinozaki E, Takeda Y, Wakatsuki T, Ichimura T, Saiura A, Yamaguchi K, Takahashi S, Noda T, Zembutsu H. 2019. Clinical relevance of circulating tumor DNA assessed through deep sequencing in patients with metastatic colorectal cancer. *Cancer Medicine* **8**:408–417 DOI 10.1002/cam4.1913.

Papanicolau-Sengos A, Aldape K. 2022. DNA methylation profiling: an emerging paradigm for cancer diagnosis. *Annual Review of Pathology* **17**:295–321 DOI 10.1146/annurev-pathol-042220-022304.

Pascual J, Attard G, Bidard FC, Curigliano G, De Mattos-Arruda L, Diehn M, Italiano A, Lindberg J, Merker JD, Montagut C, Normanno N, Pantel K, Pentheroudakis G, Popat S, Reis-Filho JS, Tie J, Seoane J, Tarazona N, Yoshino T, Turner NC. 2022. ESMO recommendations on the use of circulating tumour DNA assays for patients

with cancer: a report from the ESMO precision medicine working group. *Annals of Oncology* **33**:750–768 DOI 10.1016/j.annonc.2022.05.520.

Peneder P, Stütz AM, Surdez D, Krumbholz M, Semper S, Chicard M, Sheffield NC, Pierron G, Lapouble E, Tötzl M, Ergüner B, Barreca D, Rendeiro AF, Agaimy A, Boztug H, Engstler G, Dworzak M, Bernkopf M, Taschner-Mandl S, Ambros IM, Myklebost O, Marec-Bérard P, Burchill SA, Brennan B, Strauss SJ, Whelan J, Schleiermacher G, Schaefer C, Dirksen U, Hutter C, Boye K, Ambros PF, Delattre O, Metzler M, Bock C, Tomazou EM. 2021. Multimodal analysis of cell-free DNA whole-genome sequencing for pediatric cancers with low mutational burden. *Nature Communications* **12**:3230 DOI 10.1038/s41467-021-23445-w.

Piñerúa Gonsálvez JF, Zambrano-Infantino RDC, Benítez S. 2019. Chromoendoscopy using toluidine blue plus Lugol's solution for early diagnosis of esophageal pre-malignant lesions and superficial neoplasms in high-risk patients. *Arquivos de Gastroenterologia* **56**:41–44 DOI 10.1590/S0004-2803.201900000-01.

Poosari A, Nutravong T, Namwat W, Wasenang W, Sa-Ngiamwibool P, Ungaree-wittaya P. 2022. The relationship between P16(INK4A) and TP53 promoter methylation and the risk and prognosis in patients with oesophageal cancer in Thailand. *Scientific Reports* **12**:10337 DOI 10.1038/s41598-022-14658-0.

Qiao G, Zhuang W, Dong B, Li C, Xu J, Wang G, Xie L, Zhou Z, Tian D, Chen G, Tang J, Zhou H, Zhang D, Shi R, Chen R, Nian W, Zhang Y, Zhao J, Wen X, Xu Y, Li B, Zhang Z, Cai S, Ben X, Qi Y. 2021. Discovery and validation of methylation signatures in circulating cell-free DNA for early detection of esophageal cancer: a case-control study. *BMC Medicine* **19**:243 DOI 10.1186/s12916-021-02109-y.

Rajamäki K, Taira A, Katainen R, Välimäki N, Kuosmanen A, Plaketti RM, Seppälä TT, Ahtiainen M, Wirta EV, Vartiainen E, Sulo P, Ravantti J, Lehtipuro S, Granberg KJ, Nykter M, Tanskanen T, Ristimäki A, Koskensalo S, Renkonen-Sinisalo L, Lepistö A, Böhm J, Taipale J, Mecklin JP, Aavikko M, Palin K, Aaltonen LA. 2021. Genetic and epigenetic characteristics of inflammatory bowel disease-associated colorectal cancer. *Gastroenterology* **161**:592–607 DOI 10.1053/j.gastro.2021.04.042.

Ren J, Lu P, Zhou X, Liao Y, Liu X, Li J, Wang W, Wang J, Wen L, Fu W, Tang F. 2022. Genome-scale methylation analysis of circulating cell-free DNA in gastric cancer patients. *Clinical Chemistry* **68**:354–364 DOI 10.1093/clinchem/hvab204.

Salati M, Venetis K, Fassan M, Malapelle U, Pagni F, Sajjadi E, Fusco N, Ghidini M. 2021. ctDNA analysis in the personalized clinical management of gastroesophageal adenocarcinoma: turning hope into reality. *Future Oncology* **17**:4607–4618 DOI 10.2217/fon-2021-0228.

Salta S, Macedo-Silva C, Miranda-Gonçalves V, Lopes N, Gigliano D, Guimarães R, Farinha M, Sousa O, Henrique R, Jerónimo C. 2020. A DNA methylation-based test for esophageal cancer detection. *Biomarker Research* **8**:68 DOI 10.1186/s40364-020-00248-7.

Shahbandi A, Nguyen HD, Jackson JG. 2020. TP53 mutations and outcomes in breast cancer: reading beyond the headlines. *Trends in Cancer* **6**:98–110 DOI 10.1016/j.trecan.2020.01.007.

Sharma M, Verma RK, Kumar S, Kumar V. 2022. Computational challenges in detection of cancer using cell-free DNA methylation. *Computational and Structural Biotechnology Journal* **20**:26–39 DOI 10.1016/j.csbj.2021.12.001.

Shen SY, Burgener JM, Bratman SV, De Carvalho DD. 2019. Preparation of cfMeDIP-seq libraries for methylome profiling of plasma cell-free DNA. *Nature Protocols* **14**:2749–2780 DOI 10.1038/s41596-019-0202-2.

Shin SH, Kwon HJ, Kim HH, Min NY, Lim Y, Joo T, Lee KJ, Jeong MS, Kim H, Um SW, An C, Lee S. 2022. 23P MRE-seq based cancer screening for lung and colorectal cancer by deep learning analysis of cfDNA methylation pattern cancer screening. *Annals of Oncology* **33** DOI 10.1016/j.annonc.2022.07.050.

Sjöström M, Zhao SG, Levy S, Zhang M, Ning Y, Shrestha R, Lundberg A, Herberts C, Foye A, Aggarwal R, Hua JT, Li H, Bergamaschi A, Maurice-Dror C, Maheshwari A, Chen S, Ng SWS, Ye W, Petricca J, Fraser M, Chesner L, Perry MD, Moreno-Rodriguez T, Chen WS, Alumkal JJ, Chou J, Morgans AK, Beer TM, Thomas GV, Gleave M, Lloyd P, Phillips T, McCarthy E, Haffner MC, Zoubeidi A, Annala M, Reiter RE, Rettig MB, Witte ON, Fong L, Bose R, Huang FW, Luo J, Bjartell A, Lang JM, Mahajan NP, Lara PN, Evans CP, Tran PT, Posadas EM, He C, Cui XL, Huang J, Zwart W, Gilbert LA, Maher CA, Boutros PC, Chi KN, Ashworth A, Small EJ, He HH, Wyatt AW, Quigley DA, Feng FY. 2022. The 5-hydroxymethylcytosine landscape of prostate cancer. *Cancer Research* **82**:3888–3902 DOI 10.1158/0008-5472.CAN-22-1123.

Sozzi G, Roz L, Conte D, Mariani L, Andriani F, Verderio P, Pastorino U. 2005. Effects of prolonged storage of whole plasma or isolated plasma DNA on the results of circulating DNA quantification assays. *Journal of the National Cancer Institute* **97**:1848–1850 DOI 10.1093/jnci/dji432.

Sun T, Wu R, Ming L. 2019. The role of m6A RNA methylation in cancer. *Biomedicine and Pharmacotherapy* **112**:108613 DOI 10.1016/j.biopha.2019.108613.

Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. 2021. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians* **71**:209–249 DOI 10.3322/caac.21660.

Talukdar FR, Soares Lima SC, Khoueiry R, Laskar RS, Cuenin C, Sorroche BP, Boisson AC, Abedi-Ardekani B, Carreira C, Menya D, Dzamalala CP, Assefa M, Aseffa A, Miranda-Gonçalves V, Jerónimo C, Henrique RM, Shakeri R, Malekzadeh R, Gasmelseed N, Ellaithi M, Gangane N, Middleton DRS, Le Calvez-Kelm F, Ghantous A, Roux ML, Schüz J, McCormack V, Parker MI, Pinto LFR, Herceg Z. 2021. Genome-wide DNA methylation profiling of esophageal squamous cell carcinoma from global high-incidence regions identifies crucial genes and potential cancer markers. *Cancer Research* **81**:2612–2624 DOI 10.1158/0008-5472.CAN-20-3445.

Tanaka Y, Chiwaki F, Kojima S, Kawazu M, Komatsu M, Ueno T, Inoue S, Sekine S, Matsusaki K, Matsushita H, Boku N, Kanai Y, Yatabe Y, Sasaki H, Mano H. 2021. Multi-omic profiling of peritoneal metastases in gastric cancer identifies

molecular subtypes and therapeutic vulnerabilities. *Nature Cancer* **2**:962–977 DOI 10.1038/s43018-021-00240-6.

Tourancheau A, Mead EA, Zhang XS, Fang G. 2021. Discovering multiple types of DNA methylation from bacteria and microbiome using nanopore sequencing. *Nature Methods* **18**:491–498 DOI 10.1038/s41592-021-01109-3.

Turpin M, Salbert G. 2022. 5-methylcytosine turnover: mechanisms and therapeutic implications in cancer. *Frontiers in Molecular Biosciences* **9**:976862 DOI 10.3389/fmolb.2022.976862.

Wang M, Huang X, Li X, Guo Q, Xu W, Zhao M, Wang X, Wang L, Lou J. 2021. Performance comparison of commercial kits for isolating and detecting circulating tumor DNA. *Scandinavian Journal of Clinical and Laboratory Investigation* **81**:276–281 DOI 10.1080/00365513.2020.1821394.

Wang GQ, Jiao GG, Chang FB, Fang WH, Song JX, Lu N, Lin DM, Xie YQ, Yang L. 2004. Long-term results of operation for 420 patients with early squamous cell esophageal carcinoma discovered by screening. *Annals of Thoracic Surgery* **77**:1740–1744 DOI 10.1016/j.athoracsur.2003.10.098.

Wang S, Pan D, Su M, Huang G, Sun G. 2020. Moderately high folate level may offset the effects of aberrant DNA methylation of P16 and P53 genes in esophageal squamous cell carcinoma and precancerous lesions. *Genes and Nutrition* **15**:18 DOI 10.1186/s12263-020-00677-x.

Warton K, Lin V, Navin T, Armstrong NJ, Kaplan W, Ying K, Gloss B, Mangs H, Nair SS, Hacker NF, Sutherland RL, Clark SJ, Samimi G. 2014. Methylation-capture and next-generation sequencing of free circulating DNA from human plasma. *BMC Genomics* **15**:476 DOI 10.1186/1471-2164-15-476.

Weinberg DN, Rosenbaum P, Chen X, Barrows D, Horth C, Marunde MR, Popova IK, Gillespie ZB, Keogh MC, Lu C, Majewski J, Allis CD. 2021. Two competing mechanisms of DNMT3A recruitment regulate the dynamics of de novo DNA methylation at PRC1-targeted CpG islands. *Nature Genetics* **53**:794–800 DOI 10.1038/s41588-021-00856-5.

Wu J, Liu Z, Huang T, Wang Y, Song MM, Song T, Long G, Zhang X, Li X, Zhang L. 2023. Cerebrospinal fluid circulating tumor DNA depicts profiling of brain metastasis in NSCLC. *Molecular Oncology* **17**:810–824 DOI 10.1002/1878-0261.13357.

Wu H, Zhang Y. 2014. Reversing DNA methylation: mechanisms, genomics, and biological functions. *Cell* **156**:45–68 DOI 10.1016/j.cell.2013.12.019.

Xiao Z, Wu W, Wu C, Li M, Sun F, Zheng L, Liu G, Li X, Yun Z, Tang J, Yu Y, Luo S, Sun W, Feng X, Cheng Q, Tao X, Wu S, Tao J. 2021. 5-hydroxymethylcytosine signature in circulating cell-free DNA as a potential diagnostic factor for early-stage colorectal cancer and precancerous adenoma. *Molecular Oncology* **15**:138–150 DOI 10.1002/1878-0261.12833.

Yang J, Gong Y, Lam VK, Shi Y, Guan Y, Zhang Y, Ji L, Chen Y, Zhao Y, Qian F, Chen J, Li P, Zhang F, Wang J, Zhang X, Yang L, Kopetz S, Futreal PA, Zhang J, Yi X, Xia X, Yu P. 2020. Deep sequencing of circulating tumor DNA detects molecular residual

disease and predicts recurrence in gastric cancer. *Cell Death and Disease* **11**:346 DOI 10.1038/s41419-020-2531-z.

Yang W, Guo C, Herman JG, Zhu C, Lv H, Su X, Zhang L, Zhang M, Guo M. 2022. Epigenetic silencing of JAM3 promotes esophageal cancer development by activating Wnt signaling. *Clinical Epigenetics* **14**:164 DOI 10.1186/s13148-022-01388-3.

Yu SCY, Deng J, Qiao R, Cheng SH, Peng W, Lau SL, Choy LYL, Leung TY, Wong J, Wong VW, Wong GLH, Jiang P, Chiu RWK, Chan KCA, Lo YMD. 2023. Comparison of single molecule, real-time sequencing and nanopore sequencing for analysis of the size, end-motif, and tissue-of-origin of long cell-free DNA in plasma. *Clinical Chemistry* **69**:168–179 DOI 10.1093/clinchem/hvac180.

Yu SC, Lee SW, Jiang P, Leung TY, Chan KC, Chiu RW, Lo YM. 2013. High-resolution profiling of fetal DNA clearance from maternal plasma by massively parallel sequencing. *Clinical Chemistry* **59**:1228–1237 DOI 10.1373/clinchem.2013.203679.

Yu Q, Xia N, Zhao Y, Jin H, Chen R, Ye F, Chen L, Xie Y, Wan K, Zhou J, Zhou D, Lv X. 2022. Genome-wide methylation profiling identify hypermethylated HOXL subclass genes as potential markers for esophageal squamous cell carcinoma detection. *BMC Medical Genomics* **15**:247 DOI 10.1186/s12920-022-01401-x.

Zee A, Deng DZQ, Adams M, Schimke KD, Corbett-Detig R, Russell SL, Zhang X, Schmitz RJ, Vollmers C. 2022. Sequencing illumina libraries at high accuracy on the ONT MinION using R2C2. *Genome Research* **32**:2092–2106 DOI 10.1101/gr.277031.122.

Zhang H, Dong P, Guo S, Tao C, Chen W, Zhao W, Wang J, Cheung R, Villanueva A, Fan J, Ding H, Schrodi SJ, Zhang D, Zeng C. 2020. Hypomethylation in HBV integration regions aids non-invasive surveillance to hepatocellular carcinoma by low-pass genome-wide bisulfite sequencing. *BMC Medicine* **18**:200 DOI 10.1186/s12916-020-01667-x.

Zhang Q, Liu F, Chen W, Miao H, Liang H, Liao Z, Zhang Z, Zhang B. 2021. The role of RNA m(5)C modification in cancer metastasis. *International Journal of Biological Sciences* **17**:3369–3380 DOI 10.7150/ijbs.61439.

Zhang X, Li T, Niu Q, Qin CJ, Zhang M, Wu GM, Li HZ, Li Y, Wang C, Du WF, Wang CY, Zhao Q, Zhao XD, Wang XL, Zhu JB. 2022. Genome-wide analysis of cell-free DNA methylation profiling with MeDIP-seq identified potential biomarkers for colorectal cancer. *World Journal of Surgical Oncology* **20**:21 DOI 10.1186/s12957-022-02487-4.

Zhao J, He YT, Zheng RS, Zhang SW, Chen WQ. 2012. Analysis of esophageal cancer time trends in China, 1989–2008. *Asian Pacific Journal of Cancer Prevention: APJCP* **13**:4613–4617 DOI 10.7314/apjcp.2012.13.9.4613.

Zhou X, Cheng Z, Dong M, Liu Q, Yang W, Liu M, Tian J, Cheng W. 2022. Tumor fractions deciphered from circulating cell-free DNA methylation for cancer early diagnosis. *Nature Communications* **13**:7694 DOI 10.1038/s41467-022-35320-3.

Zhou J, Wang C, Lin G, Xiao Y, Jia W, Xiao G, Liu Q, Wu B, Wu A, Qiu H, Zhang F, Hu K, Xue H, Shen Z, Wang Z, Han J, Niu B, Xu Y, Yu Z, Yang L. 2021. Serial circulating tumor DNA in predicting and monitoring the effect of neoadjuvant

chemoradiotherapy in patients with rectal cancer: a prospective multicenter study. *Clinical Cancer Research* **27**:301–310 DOI 10.1158/1078-0432.CCR-20-2299.

Zhou M, Wang H, Zeng X, Yin P, Zhu J, Chen W, Li X, Wang L, Wang L, Liu Y, Liu J, Zhang M, Qi J, Yu S, Afshin A, Gakidou E, Glenn S, Krish VS, Miller-Petrie MK, Mountjoy-Venning WC, Mullany EC, Redford SB, Liu H, Naghavi M, Hay SI, Wang L, Murray CJL, Liang X. 2019. Mortality, morbidity, and risk factors in China and its provinces, 1990–2017: a systematic analysis for the global burden of disease study 2017. *Lancet* **394**:1145–1158 DOI 10.1016/S0140-6736(19)30427-1.

Zhou Y, Zhu J, Zhao M, Zhang B, Jiang C, Yang X. 2018. Methylation-level inferences and detection of differential methylation with MeDIP-seq data. *PLOS ONE* **13**:e0201586 DOI 10.1371/journal.pone.0201586.

Zviran A, Schulman RC, Shah M, Hill STK, Deochand S, Khamnei CC, Maloney D, Patel K, Liao W, Widman AJ, Wong P, Callahan MK, Ha G, Reed S, Rotem D, Frederick D, Sharova T, Miao B, Kim T, Gydush G, Rhoades J, Huang KY, Omans ND, Bolan PO, Lipsky AH, Ang C, Malbari M, Spinelli CF, Kazancioglu S, Runnels AM, Fennessey S, Stolte C, Gaiti F, Inghirami GG, Adalsteinsson V, Houck-Loomis B, Ishii J, Wolchok JD, Boland G, Robine N, Altorki NK, Landau DA. 2020. Genome-wide cell-free DNA mutational integration enables ultra-sensitive cancer monitoring. *Nature Medicine* **26**:1114–1124 DOI 10.1038/s41591-020-0915-3.