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Current advances in detecting genetic and epigenetic biomarkers of colorectal cancer

Md Sajedul Islam [a,b], Vinod Gopalan [a,b,**], Alfred K. Lam [a,b,c], Muhammad J.A. Shiddiky [d,*]

[a] Cancer Molecular Pathology, School of Medicine & Dentistry, Griffith University, Gold Coast Campus, Southport, QLD, 4222, Australia
[b] Menzies Health Institute Queensland, Griffith University, Gold Coast, QLD, 4222, Australia
[c] Pathology Queensland, Gold Coast University Hospital, Southport, QLD, 4215, Australia
[d] Rural Health Research Institute, Charles Sturt University, Orange, NSW, 2800, Australia

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ABSTRACT

Colorectal carcinoma (CRC) is the third most common cancer in terms of diagnosis and the second in terms of mortality. Recent studies have shown that various proteins, extracellular vesicles (i.e., exosomes), specific genetic variants, gene transcripts, cell-free DNA (cfDNA), circulating tumor DNA (ctDNA), microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and altered epigenetic patterns, can be used to detect, and assess the prognosis of CRC. Over the last decade, a plethora of conventional methodologies (e.g., polymerase chain reaction [PCR], direct sequencing, enzyme-linked immunosorbent assay [ELISA], microarray, *in situ* hybridization) as well as advanced analytical methodologies (e.g., microfluidics, electrochemical biosensors, surface-enhanced Raman spectroscopy [SERS]) have been developed for analyzing genetic and epigenetic biomarkers using both optical and non-optical tools. Despite these methodologies, no gold standard detection method has yet been implemented that can analyze CRC with high specificity and sensitivity in an inexpensive, simple, and time-efficient manner. Moreover, until now, no study has critically reviewed the advantages and limitations of these methodologies. Here, an overview of the most used genetic and epigenetic biomarkers for CRC and their detection methods are discussed. Furthermore, a summary of the major biological, technical, and clinical challenges and advantages/limitations of existing techniques is also presented.

1. Introduction

In 2020, colorectal carcinoma (CRC) ranked third in terms of diagnosis (10%) and second in terms of mortality (9.4%), accounting for more than 1.9 million new cases and approximately 1 million deaths globally (Sung et al., 2021). The overall number of fatalities from CRC is anticipated to rise by 71.5% by the year 2035 (Douaiher et al., 2017). CRC accounts for 12.6% of all cancer treatment costs, ranking second only to breast cancer (National Cancer Institute, 2022). In 2020, the total annual cost of CRC medical care in the United States alone was an astounding US$24.3 billion (American Cancer Society, 2022). CRC progresses through continuous accumulation of genetic and epigenetic changes to form pre-cancerous lesions (adenomas and serrated polyps), which can later progress into a malignant phenotype (Dekker et al., 2019). Symptoms of CRC rarely occur until the cancer is in an advanced stage, which quite often prove to be fatal for the patient. According to the American Cancer Society, the 5-year relative survival rates of colon and rectal cancers are 64% and 67% respectively, which demonstrates the significance of detecting this disease in an earlier stage (American Cancer Society, 2022).

Various genetic and epigenetic biomarkers, including specific genetic variants, transcripts, proteins, cell-free DNA (cfDNA), circulating tumor DNA (ctDNA), altered epigenetic patterns (such as DNA/histone hypomethylation, hypermethylation, or acetylation), exosomes, microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and more, are being utilized for the detection of CRC. For example, several research studies demonstrated the significance of genetic mutations of *KRAS, NRAS, BRAF,* and *PIK3CA* for the detection of CRC (Luo et al., 2021b; Therkildsen et al., 2014). Similarly, few other studies reported different advantages and limitations of ctDNAs, miRNAs, exosomes, and circulating tumor cells (CTCs) (Luo et al., 2021a; Ogata-Kawata et al., 2014; Vasantharajan et al., 2021; Vlassov et al., 2012). Various conventional

* Corresponding author. Rural Health Research Institute, Charles Sturt University, Orange, NSW, 2800, Australia.
** Corresponding author. Cancer Molecular Pathology, School of Medicine & Dentistry, Griffith University, Gold Coast Campus, Southport, QLD, 4222, Australia.
E-mail addresses: v.gopalan@griffith.edu.au (V. Gopalan), mshiddiky@csu.edu.au (M.J.A. Shiddiky).

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strategies based on target amplification, sequencing, hybridization, and immunoassay, as well as advanced analytical techniques, are currently being employed for the detection of various biomarkers associated with CRC.

Despite the availability of multiple techniques for biomarker detection in CRC, selecting a single method as a universal detection tool remains challenging due to various associated issues and insufficient evidence to definitively establish its superiority over others. For instance, although detecting CRC-specific genetic mutations (i.e., *KRAS, BRAF, PIK3CA, APC,* and *p53*) by PCR or direct sequencing are widely used, these techniques often lack sufficient sensitivity (Itonaga et al., 2016). Moreover, genetic mutations themselves are not sufficient to confirm the presence or absence of cancer condition. It is possible to recognize intra-tumor heterogeneity and forecast acquired treatment resistance using ctDNAs. However, because of the reduced degree of methylation and the limited options for downstream analyses, they are unable to accurately predict aggressive metastasis (Gobbini et al., 2020). Although genetic materials extracted from CTCs are used for predicting cancer metastasis and have greater downstream analyzing potential, CTCs are present in small amounts in the blood with a short lifespan. Furthermore, there is no standardized CTC isolation method yet and the existing isolation and enrichment strategies are very expensive and time-consuming (Ferreira et al., 2016). Even though microfluidic approach to isolating CTCs followed by genetic and/or epigenetic profiling is preferable, a significant drawback is the presence of a relatively high amount of white blood cells (WBCs) (Nagrath et al., 2007).

Several recent reviews discussed the potential of genetic and epigenetic biomarkers in cancer detection and prognosis. In one such review, Burrell et al. critically addressed the causes and subsequent consequences of genetic heterogeneity in the evolution of cancer (Burrell et al., 2013). Then, a few other reviews summarized the common genetic and epigenetic alterations in CRC along with their frequency of occurrence and their suitability as predictive, diagnostic, or prognostic biomarkers (Alves Martins et al., 2019; Kuipers et al., 2015). Munro and colleagues reviewed CRC-associated cancer stem cell markers and their role in cancer progression (Munro et al., 2018). Later, Jung et al. extensively reviewed the epigenetic modifications in CRC as well as the role of non-coding RNAs as epigenetic regulators (Jung et al., 2020). They also discussed the potential translation of these biomarkers into clinical practice. In another review, Biller et al. discussed the therapeutic potential of some of the genetic mutations associated with CRC (Biller and Schrag, 2021). Moreover, Gallardo-Gómez et al. summarized multiple omics-based studies and concluded that blood-based epigenetic biomarkers including DNA methylations and miRNAs (e.g., miR-15b, miR-18a, miR-19a, miR-19b, miR-29a, and miR-335) provide significantly higher sensitivity and specificity (Gallardo-Gómez et al., 2021). Apart from these, lots of other reviews also addressed the role of different genetic, epigenetic, circulating, and cellular biomarkers in the initiation, transition, and/or metastasis of CRC (Bi et al., 2020b; Chen et al., 2019; El Bairi et al., 2018; Fearon, 2011; Galvano et al., 2019; Kamel et al., 2022; Kudryavtseva et al., 2016; Nguyen and Duong, 2018; Osumi et al., 2019b; Tsang et al., 2014; Ye et al., 2021).

While numerous reviews and research articles have demonstrated the significance of different biomarkers and the use of individual detection methods for CRC, to date, no other article has summarized the advantages and limitations of these methods. Moreover, these studies did not provide a comparative discussion on the analytical performance of the methods as well as the major challenges associated with them. Additionally, despite significant advancement in addressing these challenges in recent years, no previous study incorporated or systemically discussed them. Thus, in this review article, we opted to explore the common genetic and epigenetic biomarkers from CRC patients and the current detection methods for profiling them as well as their associated issues and challenges. The objective of this review is to critically compare the available conventional and advanced analytical methods and propose future endeavors that could enhance the characterization and profiling of CRC.

2. Common genetic and epigenetic biomarkers in CRC

Advances in genomics, proteomics, and molecular pathology brought about several candidate biomarkers with potential clinical usage for cancer staging, carrying out genomic, epigenomic, and/or immune profiling for selected therapy, and monitoring patient prognosis. Biomarkers for CRC can be obtained through either tissue or liquid biopsies. Genetic and epigenetic biomarkers from the colon tissues comprise the tissue biopsy, whereas liquid biopsy is typically referred to CTCs, cfDNA/ctDNA, tumor-derived extracellular vesicles, autoantibodies, and tumor-educated platelets (Aboud and Weiss, 2013; Boriachek et al., 2018a; Chen et al., 2017a; Hamid et al., 2020; Liu and Locasale, 2017; Loktionov 2007, 2020). Fig. 1 represents the common biomarkers associated with CRC.

2.1. DNA/RNA/protein-based biomarkers

2.1.1. DNA-based biomarkers

Among all the biomarkers, CRC-associated genetic mutations are the most prevalent. A study reported that mutations in genes such as *MLH1*, *MLH3*, *MSH2*, *MSH3*, *MSH6*, or *PMS2*, as well as the CpG island methylation phenotype characterized by the methylation of *CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS* genes, and mutually exclusive mutations of *KRAS* and *BRAF*, have significant implications for the diagnosis and prognosis of CRC (Luo et al., 2021b). After analyzing 22 studies with 2395 patients, Therkildsen et al. observed that apart from *KRAS* exon 2, mutations in *KRAS* exons 3 and 4, *NRAS, BRAF, PIK3CA,* and/or non-functional *PTEN* can confer resistance to anti-epidermal growth factor receptor (*EGFR*) therapies in metastatic CRC (Therkildsen et al., 2014). A study by Islam et al. found the association of *FMAB* mutation in CRC development in 46.5% of the diagnosed patients (Islam et al., 2017a). Also, Imperiale et al. reported more than 50% sensitivity of a panel consisting of 21 DNA point mutations in *APC, KRAS*, and *p53* genes, and *BAT-26* deletions (microsatellite instability marker) for CRC (Imperiale et al., 2004). Several other studies, including those by Flamini et al., (2006), Guinney et al., (2015), Lin et al., (2014), Wan et al., (2019), Wang et al., (2004); Yan et al., (2017), have reported various genetic variations as potential biomarkers of CRC. It is clear that solely

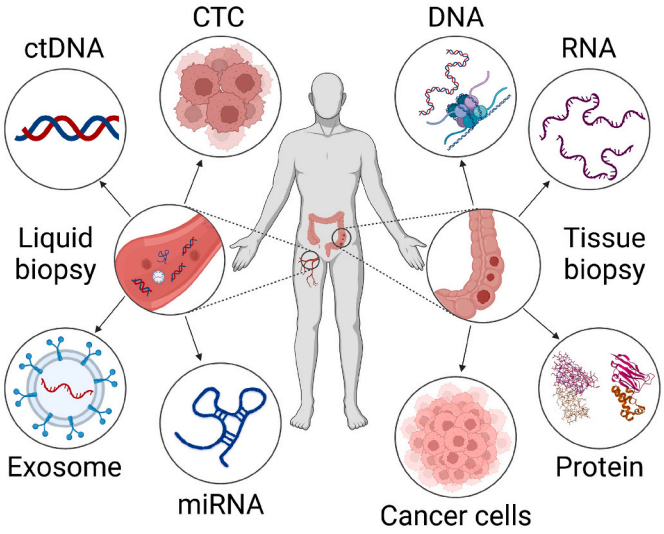

Fig. 1. Common biomarkers of colorectal carcinoma. Different DNA, RNA, proteins, circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), exosomes, and non-coding RNAs (i.e., miRNA) can serve as potential biomarkers, which can be derived through either of the tissue or liquid biopsies. This figure was generated in Biorender.

analyzing genetic mutations is inadequate for predicting cancer metastasis in diagnosed patients (Sacco et al., 2020). In this regard, a combination of multiple biomarkers would offer substantial sensitivity.

Further studies have also confirmed that DNA methylations in *BMP3*, *NDGR4*, *septin 9* (*SEPT9*), *SFRP2*, *SPG20*, *TFPI2*, and *vimentin* (*VIM*) provide diagnostic sensitivities ranging from 50 to 92% and specificity within 80–100% (Lam et al., 2016; Liu et al., 2019a; Rasmussen et al., 2016). 5-methylcytosine (5 mC) and its oxidized form, 5-hydroxymethylcytosine (5hmC), hold independent importance as biomarkers for various cancers (Li et al., 2016; Uribe-Lewis et al., 2015). Studies on CRC have been limited by small sample sizes and the limited quantitative performance of the methods used to quantify and analyze 5hmC signatures (Li et al., 2017a; Song et al., 2017a). Notably, the stage-specific loss of 5hmC in the later stages of metastatic cancer restricts its utility as a biomarker (Song et al., 2017a). Nevertheless, the inclusion of 5hmC in multianalyte testing can enhance the sensitivity for detecting early-stage CRC (Walker et al., 2022). Combining the methylation testing for *VIM*, *SFRP2*, *BMP3*, and *NDRG4*, relatively high sensitivity and specificity for CRC could be achieved (Liu et al., 2019a; Xiao et al., 2014). However, DNA methylation testing from biological samples can be challenging when presented in a point-of-care (POC) format (Jin and Liu, 2018).

Another emerging biomarker is the ctDNA, which can be screened through liquid biopsy (Dasari et al., 2020). ctDNAs are fragments of tumor DNAs that circulate through the bloodstream of a cancer patient, which comprise <1% of the cfDNA in the blood (Bi et al., 2020a). ctDNAs can be considered a surrogate for tumor biopsy, as they provide a snapshot of the tumor from which they originated (Schmiegel et al., 2017). In this context, Luo et al. highlighted the advantages and disadvantages of ctDNA profiling as well as the potential clinical applications of ctDNA methylation assays (Luo et al., 2021a). They also summarized the technologies available to analyze ctDNA methylation in cancer patients. Several studies have observed a higher level of concordance for *RAS* mutation between ctDNAs and tumors in CRC patients, indicating the feasibility of using ctDNAs as an alternative to tumor biopsy (Schmiegel et al., 2017; Vidal et al., 2017). Although ctDNA screening requires only a small amount of blood (1–2 ml), detecting ctDNAs can be challenging due to contamination with other cfDNAs and cellular debris. Nevertheless, screening for mutations in genes such as *p53*, *KRAS*, *NRAS*, and *BRAF* in ctDNAs can facilitate the identification of CRC-associated biomarkers with high specificity and sensitivity (Osumi et al., 2019a; Thierry et al., 2014; Wan et al., 2020).

Another advancement in cancer diagnosis is cfDNA fragmentomics. Typically, cfDNA in the plasma of cancer patients exhibits shorter lengths (Jiang et al., 2015; Underhill et al., 2016). Taking advantage of the size properties of cfDNA, a recent study analyzed the fragmentation profile of 236 patients with different cancers and compared them with that of 245 healthy individuals (Cristiano et al., 2019). Using a machine learning model known as DELFI, which utilized genome-wide size properties, the authors observed a sensitivity ranging from 57% to over 99% with a specificity of 98%. They also found that the size profile could help determine the tissue of origin for cancers in 75% of cases. Additionally, a review by A. R. Thierry discussed the potential usage and limitations of cfDNA fragmentomics for early cancer detection (Thierry, 2023). Moreover, Wang and colleagues presented the significance of cfDNA fragmentomics in predicting the pathological response to neoadjuvant chemoradiotherapy in locally advanced rectal cancer (Wang et al., 2022).

2.1.2. RNA-based biomarkers

Multiple genetic expression (mRNA) analyses of either a single gene or gene panel have been employed in CRC detection. Among them, the studies performed by Ardalan et al. and Wu et al. showed impressive results in terms of sensitivity (85.9–96.1%) and specificity (85.7–95%) (Ardalan Khales et al., 2015; Wu et al., 2017). Several genetic transcripts, including MMP9, GSK3A, HER2, and RHOA, have also demonstrated significant sensitivity and specificity in identifying CRC (Djaballah et al., 2022; Nichita et al., 2014; Ross et al., 2018; Xue et al., 2019).

Moreover, miRNAs are one of the important regulatory elements of gene expression and are closely related to many types of disorders including cancer (Romano et al., 2017). Multiple studies have observed varying sensitivity for CRC using miRNA variants such as miR-21, miR-223, and miR-451 from different clinical samples (Phua et al., 2014; Sazanov et al., 2017; Slaby, 2016). Combinations of upregulated miRNAs, including miR-15b, miR-18, miR-19a, miR-19b, miR-29a, and miR-335 (Herreros-Villanueva et al., 2019a), and downregulated miRNAs, such as miR-144-3p, miR-455-5p, and miR-1260b (Liu et al., 2019b), have provided significant sensitivity and specificity (>90%) for CRC. Despite the promise of miRNAs as biomarkers, further research is needed to address the relatively complex laboratory procedures and the necessity for proper assay optimization (Djaballah et al., 2022; Nichita et al., 2014; Ross et al., 2018; Xue et al., 2019).

2.1.3. Protein-based biomarkers

Alternatively, for screening and detection of cancer recurrence at surgical follow-up, measuring circulating protein biomarkers has been a good non-invasive approach (De Rubis et al., 2019). Loktionov et al. (2019) have compared 24 protein biomarkers collected non-invasively and identified that CRC could be reliably diagnosed by testing tissue inhibitors of metalloproteinase 1, peptidyl arginine deaminase 1, M2-PK, C-reactive protein (CRP), and MMP9. So far, among the detectable single protein markers in the serum, only the cysteine-rich 61 protein of the CCN family (Cyr 61), CA11-19 marker protein, B6-integrin, FAM134B, and trefoil factor 3 (TFF3) showed promising results as a biomarker for CRC (Bengs et al., 2019; Islam et al., 2017b; Nikolaou et al., 2018; Song et al., 2017b). Though protein biomarkers may accumulate in tissue and serum as cancer develops, their concentrations are usually very low, necessitating the use of highly sensitive techniques to quantify them (Diamandis and van der Merwe, 2005).

2.2. Genetic/epigenetic biomarkers from CTCs

Upon the emergence of liquid biopsy, biomolecules shed by the tumors and CTCs brought the attention of researchers. CTCs are considered minimally invasive diagnostic biomarkers for cancers, which are present significantly higher in cancer patients than in healthy people (Baek et al., 2019). CTCs are valuable biomarkers for CRC, as evidenced by research that observed outstanding diagnostic accuracy of CTCs (specificity 100% and sensitivity 99.1%) by using a microfluidic isolation platform for five different types of cancers, including CRC (Nagrath et al., 2007). Later, Vasantharajan and colleagues extensively reviewed the epigenetic landscape of CTCs (Vasantharajan et al., 2021). According to their review, aberrant methylation in the promoter regions of the tumor suppressor genes (e.g., *hMLH1*) leads to microsatellite instability and mutation in the genes. Also, hypermethylation in *SOX*17, and *CST6* genes promote metastasis, whereas hypermethylation in *hVIM,* and *SFRP2* enhances CTC plasticity and invasiveness. Furthermore, Jiang et al. thoroughly reviewed the present technologies for CTC isolation and their clinical significance in CRC (Jiang et al., 2021). Despite the promising prognostic significance of the CTCs, their use to enhance patient survival is yet to be fulfilled. The major issue regarding the isolation, characterization, and utilization of the genetic/epigenetic biomolecules of CTCs as cancer biomarkers is the presence of their minute number in the peripheral blood (1 in 10^7-10^9 cells/ml) (Sharma et al., 2018). Recent advancements in microfluidic and microchip-based platforms can achieve the isolation, purification, and enrichment of CTCs in a single chip. This can in turn minimize the need for traditional multi-step methods that usually lead to potential CTC loss (Huang et al., 2018).

2.3. Genetic/epigenetic biomarkers from exosomes

The unique composition, easier accessibility, and ability to represent the tissue of origin make the exosomes and their contents suitable biomarkers for diagnosing cancer through non-invasive methods. Exosomes carry a diverse array of biomolecules, including proteins, lipids, DNA, mRNA, and miRNAs (Vlassov et al., 2012). Various methods are typically employed to extract and isolate exosomes, such as precipitation-based approaches, gel-filtration chromatography, size-exclusion chromatography, and differential ultracentrifugation (Soda et al., 2022). Several studies have identified specific exosomal proteins (CD9, CD147) (Vlassov et al., 2012) and miRNAs (let-7a, miR-21, miR-1229, miR-1246, miR-150, miR-21, miR-223, and miR-23a) (Boriachek et al., 2018a; Ogata-Kawata et al., 2014) that can be analyzed for CRC diagnosis. However, a major challenge in the clinical application of exosomes is the shortage of reproducible and consistent techniques to extract and identify an enriched population of tumor-derived exosomes (Li et al., 2017b). Table 1 summarizes some of the major genetic and epigenetic biomarkers of CRC along with their advantages and limitations.

Table 1
Common genetic and epigenetic biomarkers of colorectal carcinoma.

Biomarker category	Biomarker type	Biomarker subtype	Advantages	Limitations	Ref.
Genetic	DNA	Mutations in *KRAS*, *NRAS, APC*, *p53, BRAF, PIK3CA, RNF43, MLH1*, *MLH3, MSH2*, *MSH3*, *MSH6*, *PMS2, FMAB*	Most common biomarker. Can estimate the risk of developing cancer.	Not sensitive enough. Presence of heterozygous alleles makes its detection complex.	(Guinney et al., 2015; Islam et al., 2017a; Lin et al., 2014; Luo et al., 2021b; Therkildsen et al., 2014; Vacante et al., 2018; Wang et al., 2004; Yan et al., 2017)
		Serum ctDNA	Requires a very small amount of blood. Provide a snapshot of the tumor from which they originated. Provides high specificity and sensitivity for cancer.	Contains huge amount of background cfDNAs and cellular debris. Does not indicate the location of the tumor.	(Flamini et al., 2006)
	RNA	Transcripts of *2CTSL1, GK, CDA, SET, PFDN5, PECAM1, APOBEC3A, UBXD5, MSL1, MMP9, BANK1, C9orf78, ISCU, DYNC1LIL2, DYM, PIP4K2B, TUG1, EPHX2, ITIH4, CDCA4, S1000A8, LST1, GSK3A, HER2, RHOA, TRIM24*	Provide dynamic insights into cellular states and regulatory processes. Multiple copies present in a cell, that renders more information than DNA. Relatively higher accuracy and specificity. Provides expression profile.	Less stable. Require sophisticated handling and storage.	(Djaballah et al., 2022; Nichita et al., 2014; Ross et al., 2018; Xue et al., 2019)
	Protein	TIMP-1, TFF3, CEA, CA11-19, IGFBP2, DKK3, PKM2, COL10A1, IL-8, Melanotransferrin, Kininogen, BAG4, IL6ST, VWF, EGFR, CD44, exosomal CD9, CD147	Gold standard for non-invasive cancer detection, screening, and surgical follow-up. Accumulate in tissue and serum as cancer develops.	Present in a very low concentration. Require extremely sensitive immunological techniques.	(Fung et al., 2015; Meng et al., 2018; Overholt et al., 2016; Rho et al., 2018; Shin et al., 2014; Solé et al., 2014; Wang et al., 2013; Xia et al., 2015; Xie et al., 2017; Yoshioka et al., 2014)
Epigenetic	DNA	Methylation of *SEPT9, CYCD2, HIC1, PAX5, RASSF1A, RB1, SRBC, ALX4, BMP3, NPTX2, ITG4, RARB, SDC2, SFRP2, VIM, C9orf50, KCNQ5, CLIP4, BCAT1, IKZF1, MLH1, TFP1,* ctDNA cg10673833	May facilitate earlier detection. Distinct cancer-specific methylation pattern can distinguish between non-neoplastic cells and cancer cells. Provide information of cancer pathological staging.	Heterogeneity of clinical samples pose a major challenge to data analysis. Lack of efficient techniques for methylation analysis.	(Cassinotti et al., 2012; Church et al., 2014; Fatemi et al., 2022; Jensen et al., 2019; Jin and Liu, 2018; Jung et al., 2020; Luo et al., 2020; Pedersen et al., 2015; Potter et al., 2014; Rasmussen et al., 2017; Ruiz-Bañobre and Goel, 2019; Song et al., 2016; Sun et al., 2019; Xie et al., 2018)
	miRNA	let-7a, miR-15a, miR-15b, miR-17, miR-18a, miR-19a, miR-19b, miR-20a, miR-21, miR-23a-3p, miR-24, miR-27a-3p, miR-29a, miR-29b, miR-31, miR-92a, miR-96, miR-106a, miR-125b, miR-135b, miR-137, miR-142–5p, miR-142-3p, miR-143, miR-145, miR-146a, miR-150, miR-194, miR-195, miR-200c, miR-221, miR-223–3p, miR-320a, miR-331, miR-335, miR-372, miR-375, miR-376c-3p, miR-421, miR-422a, miR-423-5p, miR-532-3p, miR-601, miR-625-5p, miR-652, miR-760, miR-1290, and miR-4516	Relatively stable due resistance against RNase. Can differentiate between different tumor subtypes. Distinct cancer-specific miRNA expression pattern can distinguish between non-neoplastic cells and cancer cells.	Relatively complex laboratory procedures.	(Basati et al., 2016; Carter et al. 2017a, 2017b; Chen et al. 2017b, 2023; Darroudi et al., 2023; de Groen et al., 2015; Duran-Sanchon et al., 2020; Fang et al., 2015; Herreros-Villanueva et al., 2019b; Huang et al., 2010; Hwang et al., 2014; Imaoka et al., 2016; Jin et al., 2020; Jung et al., 2020; Kanaan et al., 2013; Liu et al., 2018; Maminezhad et al., 2020; Toiyama et al., 2013; Uratani et al., 2016; Vychytilova-Faltejskova et al., 2016; Wu et al., 2014; Zheng et al., 2014)
	lncRNA	UCA1, circHIPK3, HOTAIR, CCAT1, ATB, UCA1, NEAT1, DANCR, MALAT1, MEG3, BLACAT1	Highly tissue specific and abnormally expressed in cancer.	Low specificity in distinguishing between benign and malignant tumors. May lead to inappropriate detection in moderate/low-risk patients.	(Abedini et al., 2019; Alaiyan et al., 2013; Barbagallo et al. 2018a, 2018b; Dai et al., 2017; Jung et al., 2020; Shen et al., 2020; Svoboda et al., 2014; Wu et al., 2015; Xu et al., 2011; Zhang et al., 2003)

Abbreviation: ctDNA – circulating tumor DNA; miRNA – microRNA; lncRNA – long non-coding RNA.

3. Commonly used methods for biomarker detection in CRC

Several techniques have been employed for detecting and/or quantifying the biomarkers derived from CRC patients. These techniques involve either amplification, direct sequencing, immunoassay, or hybridization strategies. The following section discusses several common methods used in detecting and/or quantifying CRC-associated biomarkers.

3.1. Amplification-based methods

Polymerase Chain Reaction (PCR)-based techniques are the most widely used, easy-to-operate methods with minimum cost for amplification of nucleic acids. Over the past decades, various PCR-based methods have been employed to selectively amplify target DNA and identify specific mutations of colorectal origin. Back in 1995, Hardingham and colleagues developed an immunobead-PCR method using immunomagnetic beads and PCR to detect CTCs as a sensitive prognostic marker for the relapse of CRC (Hardingham et al., 1995). They isolated CTCs from the blood by using immunomagnetic beads tagged with epithelial-specific antibody Ber-EP4. These CTCs were then subjected to PCR analysis to selectively amplify and detect *KRAS* codon 12 mutation. Their research obtained a sensitivity of one tumor cell/10^6 WBCs. Later, differentially expressed mature miRNAs were identified by using quantitative real-time PCR (qPCR) (Bandrés et al., 2006). Following RNA extraction and cDNA synthesis, the researchers employed qPCR with specific probes against miRNAs to determine the threshold cycle (Ct) and identified the differentially expressed miRNAs. Another study used real-time quantitative reverse transcriptase PCR (RT-qPCR) as a measure for biomarker selection for the detection of occult tumor cells in the lymph of CRC patients (Ohlsson et al., 2006). This method involved simultaneous reverse transcription of target mRNAs and real-time analysis.

Currently, various other modified PCR-based strategies have been developed for detecting CRC-associated biomarkers. For the detection of genotype and genetic mutations, allele-specific PCR (also termed as amplification refractory mutation system [ARMS]) (Kloten et al., 2017; Spindler et al., 2015; Sundström et al., 2010), custom-designed co-amplification at lower denaturation temperature (COLD-PCR) (Mancini et al., 2010a; Zuo et al., 2009), High Resolution Melt (HRM) analysis (Mancini et al., 2010b), and PCR-single strand conformation polymorphism (PCR-SSCP) (Onouchi et al., 2008) are used. The ARMS method employs allele-specific primers to specifically amplify the mutational allele sequences (Tsang et al., 2014). COLD-PCR can selectively amplify low-abundant DNA variants from a DNA pool consisting of variants and wild-type sequences, irrespective of mutation type or position on the amplicon (Li et al., 2008). PCR-SSCP can detect insertion/deletion/rearrangements in the PCR-amplified sequence followed by denaturation and gel electrophoresis in a non-denaturing polyacrylamide gel (Kakavas et al., 2008). Some cheaper alternatives to conventional PCR-based detection of genetic mutations are the single nucleotide primer extension (SNaPshot) assay and reverse hybridization (StripAssay) (Fariña Sarasqueta et al., 2011; Lewandowska et al., 2013; Magnin et al., 2011). SNaPshot employs primers with predetermined lengths targeting the genomic region with the mutation and ending at a nucleotide prior to the mutation. Fluorochrome-labeled dideoxynucleotides are then added followed by capillary electrophoresis, where the products are separated according to their sizes. Depending on the allele status (homozygous/heterozygous) of the patient, either one or two of the fluorochromes will be detected (Fig. 2a) (Fariña Sarasqueta et al., 2011). All these methods have a varying sensitivity of detecting 1–10% of the mutant sequence from the wild type population (Zhu et al., 2021).

Digital PCR (dPCR) is another robust method that can detect point mutations in ctDNA from CRC patients (Denis et al., 2016; Liebs et al., 2019; Quan et al., 2018; Taly et al., 2013b). This method also comprises the droplet-based system and microfluidic platforms for parallel PCR, such as droplet digital PCR (ddPCR) and beads, emulsification, amplification, and magnetics (BEAMing) (Taly et al., 2013a; Volik et al., 2016). ddPCR involves the partitioning of samples into thousands of nanosized droplets containing target biomarkers (e.g., ctDNA). The generated droplets then undergo PCR amplification followed by signal detection by reading fluorescence signals from probes that specifically bind to the target (Fig. 2b) (Gezer et al., 2022). In contrast, BEAMing

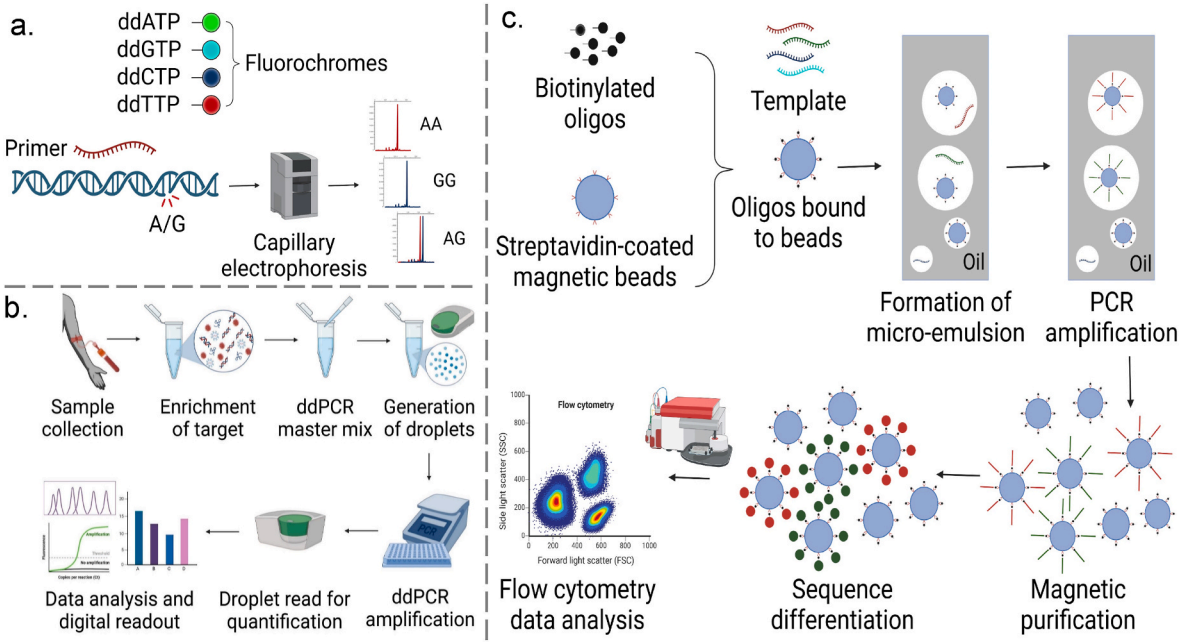

Fig. 2. Schematic representations of amplification-based detection methods. a. SNaPshot assay. Reproduced via Biorender from Ref (Fariña Sarasqueta et al., 2011). with permission from Elsevier, copyright [2011]. b. Droplet digital PCR. Reproduced via Biorender with permission under a Creative Commons Attribution License (CC-BY) from Ref (Gezer et al., 2022). copyright [2022] the authors. c. BEAMing. Reproduced via Biorender from Ref (Dressman et al., 2003). with permission from the National Academy of Sciences, U. S. A., copyright [2003].

involves attaching biotinylated oligonucleotides to magnetic beads followed by generation of microemulsions. Microemulsions are then broken up and the beads are purified using a magnet. Incubation of beads with probes differentiates distinct sequences from different templates. Bound hybridization probes are then labeled with fluorescently labeled antibodies and counted using flow cytometry (Fig. 2c) (Volik et al., 2016).

On the other hand, methylation-specific PCR (MS-PCR) (Church et al., 2014; Jensen et al., 2019; Luo et al., 2020; Pedersen et al., 2015; Potter et al., 2014; Rasmussen et al., 2017; Ruiz-Bañobre and Goel, 2019; Song et al., 2016; Sun et al., 2019; Xie et al., 2018), pyrosequencing (Irahara et al., 2010), and reverse transcriptase real-time PCR (RT-qPCR) (Chen et al., 2005) are used to detect epigenetic biomarkers. RT-qPCR can also be used to detect specific cancer-associated gene expression levels in real-time. MS-PCR is specific for the detection of DNA methylation patterns in the CpG islands (Ku et al., 2011). For this, two primer sets are used to differentiate the methylated and unmethylated DNAs upon PCR. Conventional PCR-based techniques involve different temperatures ranging from 50 to 95 °C, which calls for employing a sophisticated thermal cycler. On the contrary, isothermal amplification methods, such as Loop-mediated Isothermal Amplification (LAMP) that require a single temperature (65 °C), have been utilized for the detection of CRC-associated biomarkers (Chen et al., 2020; Lin et al., 2019). This can ultimately reduce amplification time and resource requirements. Furthermore, conventional PCRs can often result in high error rates while presenting limited performance towards detecting low allelic fraction (AF) alterations (Soda et al., 2019).

3.2. Sequencing-based methods

Another efficient approach to identifying CRC-associated genetic alterations is to sequence either specific genes or a patient's whole genome. Next Generation Sequencing (NGS), also known as massively parallel sequencing technology, provides ultra-high throughput within a short time (Fig. 3a). Several NGS platforms are currently available, including tagged-amplicon deep sequencing (TAm-seq), safe-sequencing system (Safe-SeqS), cancer personalized profiling by deep sequencing (CAPP-Seq), and methylated CpG tandem amplification and sequencing (MCTA-Seq) (Forshew et al., 2012; Li et al., 2019; Melo et al., 2014; Newman et al., 2014; Yeo et al., 2012). These platforms can analyze

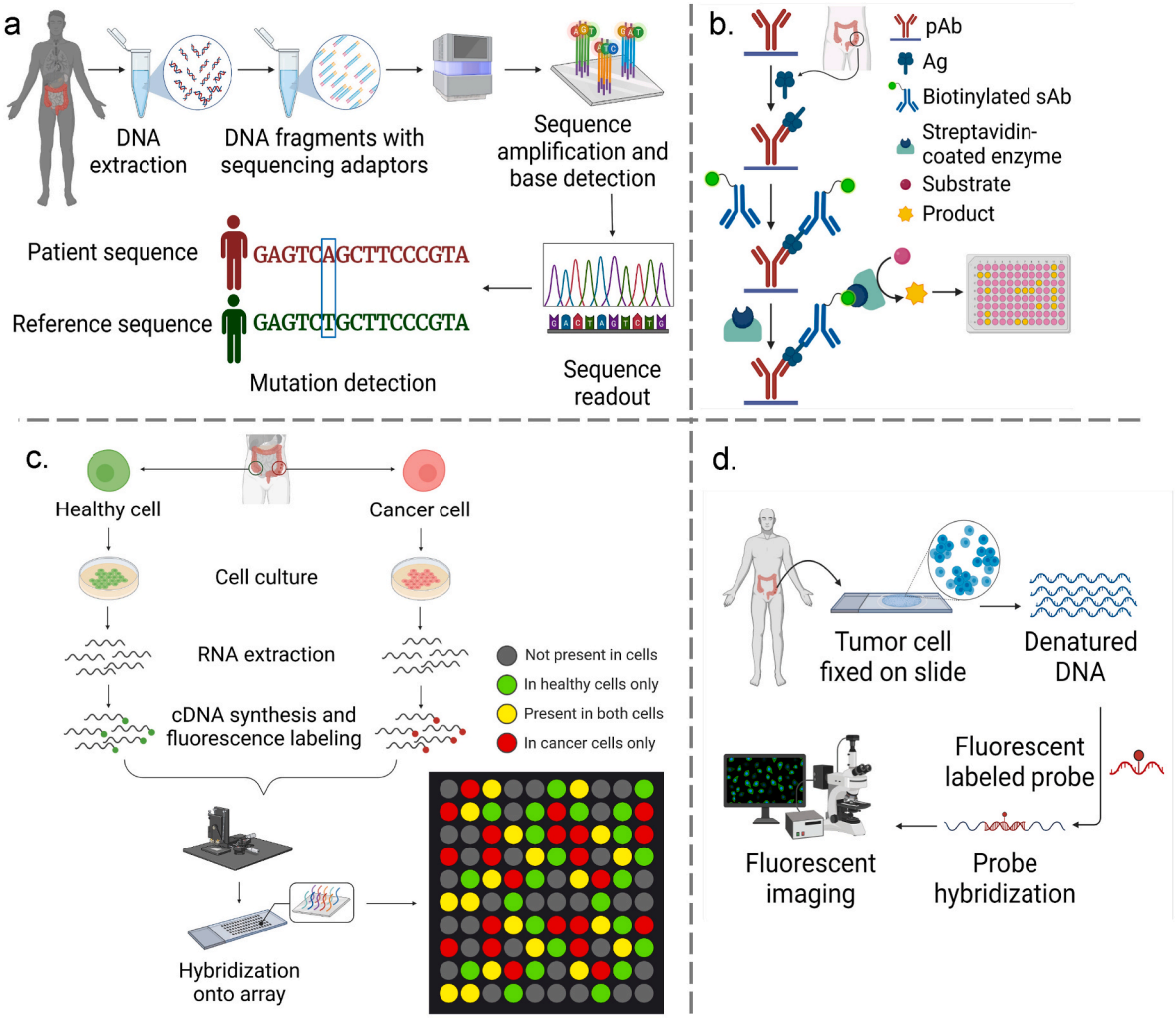

Fig. 3. Schematic workflow of different biomarker detection methods. a. Next generation sequencing. Extracted genomic DNA/cDNA are sheared to ~100–500 bp and ligated to sequencing adaptors. Following sequencing, DNA fragments are brought together and aligned either to a reference sequence or *de novo* to detect mutations. b. Enzyme-linked immunosorbent assay. Target antigens (Ag) are bound to immobilized primary antibodies (pAb) followed by binding with biotinylated secondary antibodies (sAb). Streptavidin-coated enzyme then interacts with biotin and catalyzes the transformation of specific substrate into chromogenic products, which can be visualized by colorimetric changes. c. cDNA microarray. cDNA derived from both non-neoplastic and tumor cells are fluorescently labeled and hybridized onto an array containing predetermined DNA sequences. Differential gene expression analysis is done based on the type and intensity of the fluorescence. d. Fluorescent *in situ* hybridization. Specific fluorescent-labeled probes are used against target DNA to determine the presence and location of them *in situ*. All the figures were generated in Biorender.

several millions of short DNA sequences parallelly and can either align the sequences to a reference genome or assemble *de novo* (Elazezy and Joosse, 2018; Forshew et al., 2012; Newman et al., 2014). Present NGS technologies can perform sequencing from a minute amount of DNA from a single cell. Using NGS, Strickler and colleagues studied the genetic landscape of cfDNA from CRC patients (Strickler et al., 2018). Interestingly, they observed a novel mutation in *EGFR* extracellular domain. Moreover, the latest NGS technologies can sequence from a single DNA molecule without prior amplification of the genomic library (Slatko et al., 2018). Through whole genome sequencing (WGS), the entire genome of a CRC patient can be sequenced and mapped back to the reference human genome to identify novel mutations with the full coverage of the genome (Ng and Kirkness, 2010). Similarly, whole exome sequencing (WES) can be used to map the exons and identify the genes responsible for CRC (Rabbani et al., 2014).

RNA-sequencing (RNA-seq) is yet another NGS technique that analyzes the cellular transcriptome by revealing the presence and quantity of RNA (Chu and Corey, 2012). RNA-seq is more robust in terms of identifying novel disease pathogenesis, profiling biomarkers for clinical confirmation, and offering genetic diagnoses that can be extrapolated into a personalized setting. Earlier, Seshagiri et al. identified multiple fusion transcripts including recurrent gene fusions (i.e., *RSPO2* and *RSPO3*) using RNA-seq data (Seshagiri et al., 2012). Using RNA-seq, several novel configurations of gene fusions associated with CRC, such as *BRAF*, *NTRK3*, and *RET*, have been identified, which can enhance the progression of malignancy (Kloosterman et al., 2017). More recently, a study was conducted using RNA-seq that observed circRNA_0001178 and circRNAs_0000826 biomarkers associated with the metastasis of CRC to the liver (Xu et al., 2019). lncRNAs such as RAMS11 were also found to be potential biomarkers for CRC diagnosis and therapeutic intervention (Silva-Fisher et al., 2020). One major limitation of sequencing is the lack of high sensitivity in detecting specific point mutations (Anderson, 2011). Since clinical samples contain heterogenous cells (wild type and mutated), sequencing is not sensitive enough to differentiate between them (Tucker et al., 2009).

Alternatively, bisulfite conversion is considered the gold standard for determining the methylation status of DNA. After treatment with sodium bisulfite, unmethylated cytosine residues are converted to uracil during PCR amplification, while the methylated cytosine residues remain unchanged (Grunau et al., 2001). Combining bisulfite conversion with DNA sequencing enables qualitative and quantitative detection of methylated cytosine at the single-base pair level. Treating bisulfite-converted DNA with restriction enzymes (such as *Bst*UI) followed by electrophoresis can be used to approximate the rate of cytosine methylation (Yang et al., 2004). Bisulfite sequencing (BS-seq) delivers more robust results, particularly for trace amounts of DNA with poor quality, and allows for better CpG coverage with single-base precision (Tanić, 2020). However, improper conversion of 5-methylcytosine to thymidine and failure to convert unmethylated cytosine to uracil may lead to false positive or negative results. Traditional bisulfite conversion may also require prolonged exposure of the DNA to extreme pH and temperature, resulting in DNA degradation (Tanaka and Okamoto, 2007).

To address these issues, methylation-dependent restriction enzymes (such as MspJI and AbaSI) have been used to detect 5 mC and 5hmC (Cohen-Karni et al., 2011; Mooijman et al., 2016). Another enzymatic method for identifying 5hmC is ACE-seq, which relies on the enzymes T4-phage beta-glucosyltransferase (T4-BGT) and apolipoprotein B mRNA editing enzyme catalytic subunit 3A (APOBEC3A) (Schutsky et al., 2018). In this method, T4-BGT glycosylates 5hmC, preventing its deamination by APOBEC3A, which converts unmethylated cytosines and 5 mC to thymines. A purely enzymatic treatment method for the detection of 5 mC and 5hmC is enzymatic methyl sequencing (EM-seq) (Vaisvila et al., 2021). In EM-seq, Tet methylcytosine dioxygenase 2 (TET2) and T4-BGT protect 5 mC and 5hmC from deamination by APOBEC3A, resulting in their discrimination during sequencing.

3.3. Immunoassay methods

Enzyme-linked Immunosorbent Assay (ELISA) is a well-known quantitative analytical technique that represents antigen-antibody interactions through colorimetric changes achieved by incorporating enzyme-linked conjugates and enzyme substrates (Fig. 3b). This can detect and quantify the concentration of molecules in the biological fluids (Engvall and Perlmann, 1971; Van Weemen and Schuurs, 1971). Identifying the autoantibodies produced against tumor proteins in the serum, antigens specific for certain cancers can be quantified indirectly. ELISA can also be utilized to analyze various circulating biomarkers as well. By employing ELISA, Higginbotham et al. detected the contents of the exosomes derived from a colon cancer cell line DLD-1 containing wild-type and a *KRAS* mutant allele (Higginbotham et al., 2011). They observed that mutant *KRAS* allele (DKs-8) status correlates with increased exosomal amphiregulin (AREG) levels and invasiveness of recipient cells compared to the wild-type counterpart (DKO-1). Another study used the proteomic approach and found that serine/threonine kinase 4 (STK4/MST1) expression level was lower in CRC patients compared to healthy control subjects (Yu et al., 2017). Likewise, Wang et al. identified four novel serum autoantibodies (ALDH1B1, UQCRC1, CTAG1, and CENPF) that can be used as early detection CRC biomarkers (Wang et al., 2020). Boriachek et al. (2019) developed a direct colorimetric and electrochemical ELISA-type method for CRC biomarker detection using gold-loaded nanoporous ferric oxide nanozymes functionalized with anti-CD9/CD63 antibodies. Although ELISA is relatively cheaper and has standardized protocols, it is more time-expensive, and require extra care while handling (Mascini and Tombelli, 2008). Moreover, they have lower detection limit and often interact non-specifically (Nimse et al., 2016).

3.4. Hybridization-based methods

Hybridization-based gene expression analysis, especially microarray, is a well-known method for detecting various biomarkers associated with CRC. Microarray enables the simultaneous detection of the expression levels of thousands of genes (Fig. 3c). In a review article, Li and colleagues discussed different technologies that can be employed for miRNA detection using microarrays (Li and Ruan, 2009). By profiling the serum of CRC patients using a novel glycopeptide microarray, Pedersen et al. observed that post-translational modifications, particularly O-linked glycosylation, could be attributed to early CRC diagnosis (Pedersen et al., 2011). Pedersen et al. also found that autoantibodies of the IgA isotype against glycosylated peptides provided cancer-specific signatures. Another study observed the tumor suppressive role of miR-133a in CRC progression using a miRNA array and confirmed that miR-133a is downregulated in primary human CRC and colon cancer cell lines (Dong et al., 2013). Although microarrays can analyze numerous genes simultaneously, it is still a daunting task to pinpoint the most relevant genes from the generated high-throughput microarray data due to the lack of sufficient follow-up information for each gene. Moreover, in case of protein microarrays, instability of the protein on the solid surface and non-specific interactions due to hydrophobic surface often raise some challenges (Nimse et al., 2016).

In situ hybridization (ISH) is another robust method that can localize specific DNA or RNA sequences (Gall and Pardue, 1969). Previously, several research focused on identifying CRC-specific sequence and gene expression alterations. For example, Saito et al. utilized ISH for extensive screening of the overexpressed genes in CRC (Saito et al., 2002). Another study by Baker and colleagues developed a novel RNA-specific *in situ* mutation detection method employing ISH (Baker et al., 2017). The study could distinguish among the single nucleotide polymorphisms (SNPs)/variants of cancer-specific genes *in situ* and thereby minimize the challenges associated with intra-tumor heterogeneity. Later, modified versions of this technique, i.e., chromogenic *in situ* hybridization (CISH) and fluorescent *in situ* hybridization (FISH) came into play. Whereas

CISH utilizes probes with chromogenic enzymes (i.e., Alkaline phosphatase [AP], Horseradish peroxidase [HRP]), FISH uses fluorescent labels allowing for multiplexing and signal quantification (Fig. 3d) (Sasi et al., 2021). Using CISH, Shia et al. investigated the status of *EGFR* expression patterns in CRC (Shia et al., 2005). Although FISH is more robust, it cannot detect miRNAs with very low copy number (Zhang et al., 2015). However, unlike RT-qPCR and microarray, ISH preserves the spatial information that can be used to investigate the regulatory networks within a tumor micro-environment (Sasi et al., 2021).

Table 2 summarizes the advantages and limitations of the commonly used methods for the detection of CRC-associated biomarkers.

4. Advanced analytical methods for biomarker detection in CRC

Over the last few decades, advances in analytical methods have brought about a paradigm shift in the diagnosis, prognosis, and treatment of cancer-associated disorders. Several major analytical applications in cancer biology have been validated, including early cancer screening and detection, as well as the development of novel therapy techniques that cannot be obtained via existing conventional methods (Laroui et al., 2013). In this context, recently, Brar and colleagues published an extensive literature review on the role of nanotechnology in precision diagnosis and therapy of CRC (Brar et al., 2021). In this section, current advanced analytical methodologies relevant to the detection of biomarkers of CRC are discussed briefly.

4.1. Microfluidics-based methods

Microfluidics-based methods allow *in vitro* cell sorting, quantification, and single-cell analysis while enabling fast and inexpensive

Table 2
Summary of commonly used detection methods for CRC.

Detection principle	Method	Advantages	Limitations	Ref.
Amplification	qPCR ARMS PCR-SSCP COLD-PCR HRM	Provides faster and high-throughput detection and quantification of targets in real-time. Inexpensive. Time efficient.	Results need to be correlated with phenotypic and biochemical tests to confirm. High amplification error-rates. Only detect limited genomic loci amplification.	(Soda et al., 2019)
	RT-PCR	High sensitivity due to exponential amplification of RNA template. It can detect the target transcript with a low copy number.	Chances of false negative result due to RNA degradation. Limiting factor involves deficient expression of marker genes in tumors.	(Nichita et al., 2014)
	RT-qPCR	Large dynamic range (6 orders of magnitude), easier automation, large amount of sample can be processed. Reduced postamplification handling.	Presence of some amplification inhibitors may lead to false negative results. Expensive and difficult to scale.	(Jung et al., 2020)
	MS-PCR	Simple, rapid, and cost-effective. Can detect DNA methylation status of any CpG group from a very small amount of DNA. Doesn't rely on methylation-specific restriction enzyme.	Possibility of false positive and variable results due to assay conditions. Highly sequence context dependent, which often leads to overestimation of methylation.	(Jung et al., 2020)
	dPCR ddPCR BEAMing	High sensitivity.	Only detect limited genomic locus.	(Galbiati et al., 2019)
Isothermal amplification	LAMP RT-LAMP RPA	Operational in a single temperature. High sensitivity and specificity. Requires simple laboratory procedure. Requires no expensive thermocycler.	Less versatile than PCR. Less downstream analysis ability. Lacks multiplexing capability.	(Daunay et al., 2019; Gadkar et al., 2018)
Sequencing	WGS WES	Delivers high-throughput data within a short time. Detects SNPs, indel variants, copy number changes, and large structural changes.	Requires reference sequences for both wild type and variants to identify sequence abnormalities.	(Yan et al., 2017)
	NGS TAm-seq Safe-SeqS CAPP-Seq MCTA-Seq	Highly sensitive for target sequence. Cost effective.	Less sensitive in detecting low allelic frequency. Less comprehensive compared to WGS.	(Anderson, 2011; Noguchi et al., 2020; Ståhlberg et al., 2016)
	RNA-seq	Independent of previous sequence information. Does not rely on hybridization. Can detect alternative splicing when aligned to the genome. Useful for SNP identification.	Requires high power computing facilities thereby increasing cost significantly. Analysis of splice variants is quite complex.	(Djaballah et al., 2022; Ross et al., 2018; Xue et al., 2019)
Immunoassay	ELISA	Efficient sensitivity and specificity due to antibody-antigen reaction and cost-effective.	Narrow dynamic range, high background, labor intensive and time consuming. Lacks scalability and can't detect weak protein-protein interactions.	(Mascini and Tombelli, 2008; Nimse et al., 2016)
Hybridization	Microarray	Well defined protocol with standardized approaches for data submission.	Can analyze pre-defined sequences only. Reliant on hybridization, which may be non-specific. High variance for low expressed genes.	(Cassinotti et al., 2012; Nimse et al., 2016; Rho et al., 2018)
	ISH CISH FISH	Distinguish between SNPs. Can investigate the regulatory networks within a tumor micro-environment.	Cannot detect miRNAs with very low copy number.	(Sasi et al., 2021; Zhang et al., 2015)

Abbreviation: qPCR – quantitative real-time polymerase chain reaction; ARMS – amplification refractory mutation system; PCR-SSCP – PCR-single strand conformation polymorphism; COLD-PCR – co-amplification at lower denaturation temperature; HRM – high resolution melt; RT-PCR – reverse transcriptase PCR; RT-qPCR – reverse transcriptase real-time PCR; MS-PCR – methylation-specific PCR; WGS – whole genome sequencing; WES – whole exome sequencing; SNP – single nucleotide polymorphism; Indel – insertion/deletion; NGS – next generation sequencing; TAm-seq – tagged-amplicon deep sequencing; Safe-SeqS –safe-sequencing system; CAPP-Seq – cancer personalized profiling by deep sequencing; MCTA-Seq – methylated CpG tandem amplification and sequencing; ELISA – enzyme-linked immunosorbant assay; ISH – *in situ* hybridization; CISH – chromogenic *in situ* hybridization; FISH – fluorescence *in situ* hybridization.

detection by analyzing low amounts of samples and reagents in the channels (Zare and Kim, 2010). Several microfluidic platforms have been developed to efficiently detect cancer-associated biomarkers (i.e., CTCs, and exosomes) followed by subsequent genetic/epigenetic profiling. In 2007, Nagrath et al. developed the first ever microfluidic device exclusive for CTC enrichment using microfabrication methods (Nagrath et al., 2007). As precise control of fluid flow is required for efficient capture of the cells, the researchers prepared a CTC-chip consisting of 78,000 mircoposts, which were chemically functionalized with anti-EpCAM antibodies (targeting cytokeratin of carcinoma cells). The major drawback of their design was the low fluid flow rate of 1–2 ml/h, resulting in a low throughput rate. However, various modifications enhanced the capturing capability of these devices. Following the implementation of geometrically enhanced differential immunocapture that minimizes non-specific leukocyte adhesion (Galletti et al., 2014), Mikolajczyk et al. used a tumor-associated antibody cocktail along with anti-EpCAM, which was then commercialized by Biocept as OncoCEE (Mikolajczyk et al., 2011). To circumvent the limitations of the micropost-based assays requiring surface chemistry modifications, a number of other studies employed the strategy of developing surface-capture microfluidic devices (Sheng et al., 2014; Stott et al., 2010). In place of the micropost-arrays, these devices utilized antibody-coated surfaces to enhance CTC capture. Yet still, challenges arose as the captured CTCs were immobilized and could not be used for further downstream analyses. To overcome this issue, researchers used immunomagnetic technologies and immobilized CTCs on magnetic beads (Autebert et al., 2015; Saliba et al., 2010). A study in 2014 proposed a microfluidic approach enabling on-chip immunoisolation and protein analysis of circulating exosomes *in situ* from patient plasma (He et al., 2014). The PDMS-based chip employed a cascading microchannel circuit to sequentially perform exosome isolation and enrichment, chemical lysis, and immunoprecipitation of exosomal contents, and chemifluorescence-based sandwich immunoassay (Fig. 4a).

Negative enrichment technologies are usually employed to minimize

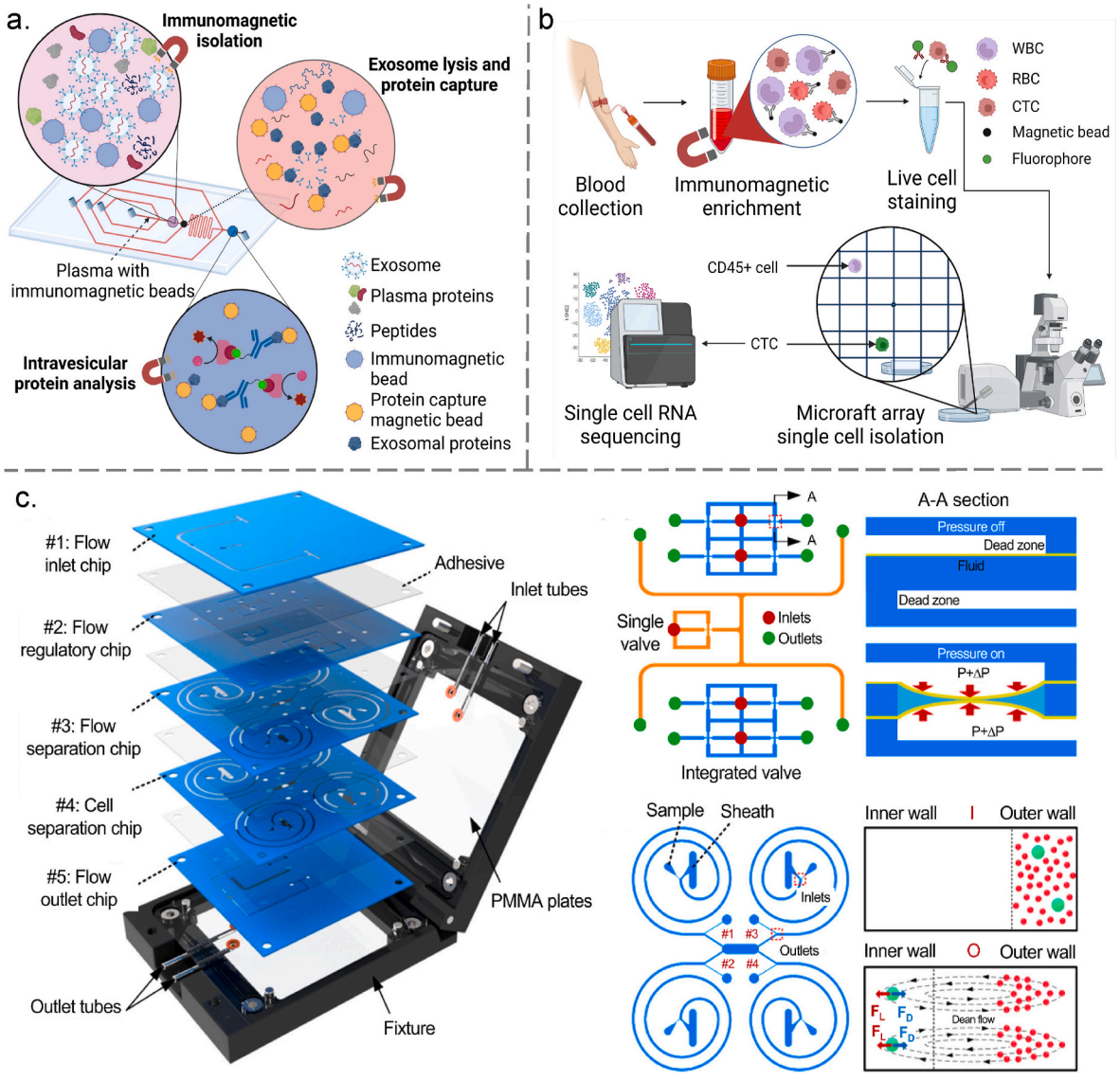

Fig. 4. Schematic representation of microfluidics and immunomagnetic-based methods. a. Integrated multi-stage microfluidic exosome analysis from human plasma. Multi-stage exosome analysis cascading microchannel network on a prototype PDMS device with on-chip immunomagnetic separation, chemical lysis, and intravesicular protein analysis of circulating exosomes. Reproduced via Biorender from Ref (He et al., 2014). under a Creative Commons Attribution License (CC-BY-NC) from the Royal Society of Chemistry, copyright [2014]. b. CTC isolation using STEMCELL Technologies EasySep® direct human CTC enrichment. Reproduced in Biorender with permission under a Creative Commons Attribution License (CC-BY) from Ref (Fankhauser et al., 2022). copyright [2022] the authors. c. Label-free automated microfluidic device. Particles are separated in the spiral channel outlets by the inertial lift force and Dean drag force. Adapted and reproduced from Ref (Zhang et al., 2018). with permission from the American Chemical Society, copyright [2018].

leukocyte contamination within the sample. One such platform is the commercialized EasySep® system developed by STEMCELL Technologies (Fig. 4b) (Liu et al., 2011). This commercial kit utilizes the CD45 depletion kit containing magnetic nanoparticles and tetrameric antibody complexes that target human CD45. Using a magnet, the unlabeled cells (CTCs) are then separated from the labeled cells. More recently, Janssen Diagnostics licensed the CTC-iChip, which employs immunomagnetic CTC enrichment technologies (Karabacak et al., 2014). This device permits the processing of whole blood, where a micropillar array sorts the mononuclear cells from red blood cells (RBCs) and platelets based on their size. Later, using inertial focusing, the larger cells are arranged in a single file, which are then subjected to magnetophoresis, where CTCs are immunomagnetically separated from the other cells through positive or negative enrichment methods. This can obtain a faster processing rate (8 ml/h) compared to other similar technologies.

In 2018, an automated microfluidic instrument was developed for the first time to perform high-throughput size-based cancer cell separation in a label-free manner (Fig. 4c) (Zhang et al., 2018). The device is functionalized with a fully integrated microfluidic machinery along with a set of sturdy fluid-driven control units. The key components of this device consist of a flow regulatory chip and two cell separation chips that maintain high-throughput cell separation. One major drawback of this device is the need for sample dilution to prevent cell-cell interactions.

As mentioned earlier, dPCR and ddPCR are microfluidic approaches used in amplification-based methods to detect genetic biomarkers associated with CRC. Both ddPCR and BEAMing demonstrate exceptional sensitivity in detecting mutant sequences (0.01–0.05%) from a wild-type pool (Zhu et al., 2021). However, dPCR has several limitations, including technical complexity, expense, and reduced dynamic range compared to traditional PCR (Jones et al., 2016). Consequently, these limitations restrict its clinical utility. Although microfluidics-based methods provide various benefits in automation and rapid analysis, they are still limited to batch-to-batch variations and elevated false positive results from non-specific interactions (Chin et al., 2012).

4.2. Electrochemical biosensors

Electrochemical detection methods have recently gained much attention due to their high sensitivity and specificity for biomolecules, faster response, miniaturization abilities, and cost-effective analysis (Xu et al., 2018; Zhang et al., 2021). A recognition element, typically an antibody, interacts with the target molecules (i.e., antigens present on the exosomes) selectively, while electroactive molecules are labeled

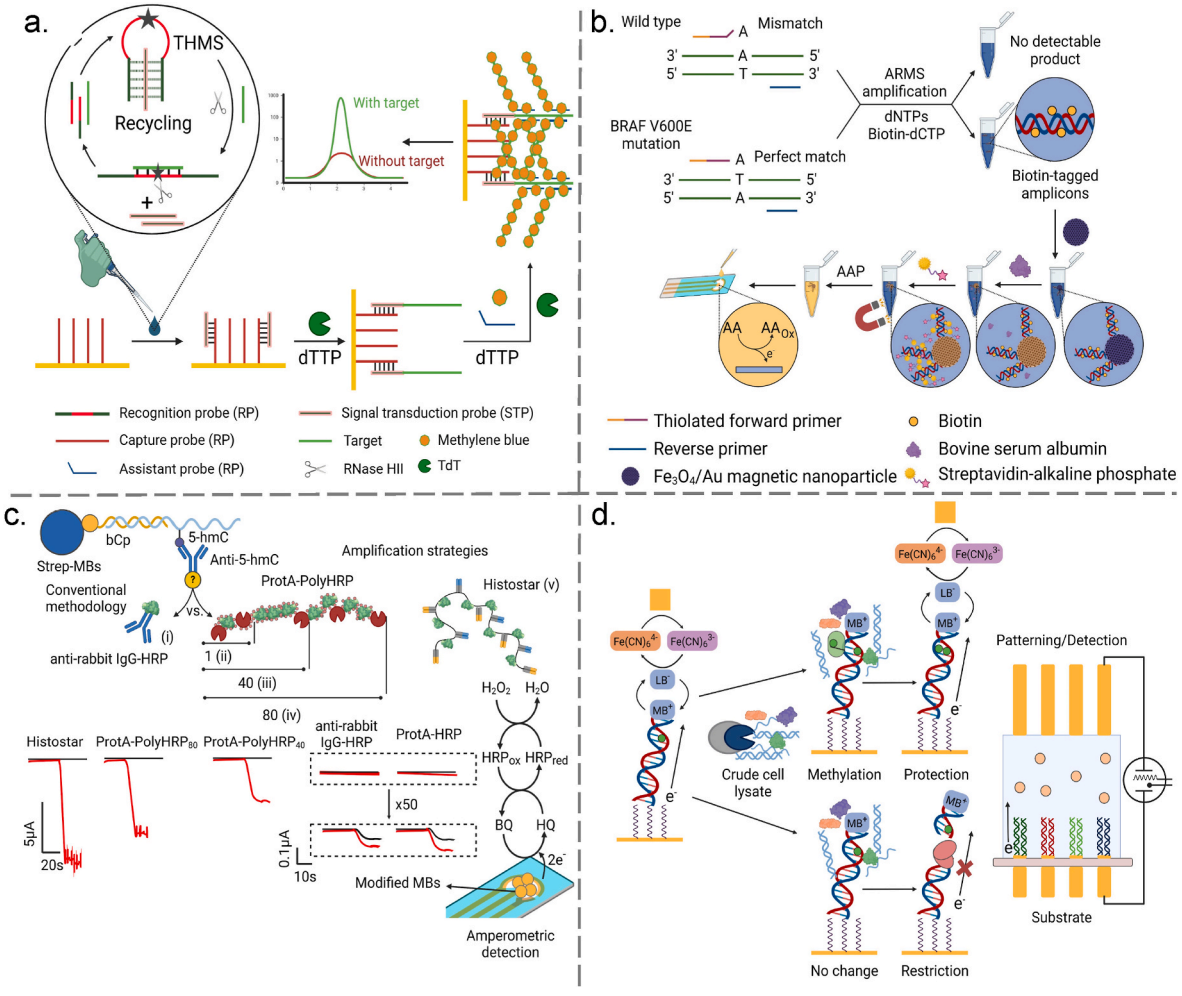

Fig. 5. Schematic representation of electrochemical biosensor-based detection methods. a. Dual enzyme-assisted multiple amplification biosensor. Reproduced in Biorender from Ref (Wang et al., 2018). with permission from Elsevier B. V., copyright [2018]. b. Electrochemical assay for sensing BRAF V600E mutation using amplification-refractory mutation system. Reproduced in Biorender from Ref (Situ et al., 2013). with permission from Elsevier, copyright [2013]. c. Amperometric bioplatforms using anti-5-hmC detector antibody enzymatic labeling strategies for synthetic DNA with 5-hmC at a specific locus. Amperometric signals obtained by applying each labeling approach for 0.0 (black lines) and 5.0 nM (red lines) of the synthetic target 1 × 5-hmC-MGMT. Reproduced in Biorender from Ref (Povedano et al., 2020). with permission from American Chemical Society, copyright [2020]. d. Electrochemical detection of human methyltransferase from crude lysate. Reproduced in Biorender from Ref (Furst et al., 2014). with permission from the National Academy of Sciences, USA, copyright [2014].

with a detection antibody that acts as an electroactive transducer (Zhou et al., 2016). Several electrochemical methods have been devised for detecting cancer-associated genetic biomarkers. Wang and colleagues developed the first electrochemical biosensor to determine the association of the *KRAS* gene with CRC (Wang et al., 2008). Thiolated capture DNA probes specific for *KRAS* were chemically adsorbed on the gold electrode that allowed the electroreduction of H_2O_2 catalyzed by horseradish peroxidase (HRP). With the help of hydroquinone as a mediator, the generated current was measured amperometrically. Later, Xiaoying Wang and colleagues reported a sandwich-type genosensor with nanofibers of carboxylated multi-walled carbon nanotubes, nylon 6, and thionine to electrostatically immobilize single stranded DNA1 (ssDNA1) capture probe (Wang et al., 2014). The target *KRAS* gene from colon cancer cells (SW480) simultaneously hybridized with ssDNA1 and gold nanoparticle-tagged ssDNA2 signal probe (AuNP-ssDNA2). Reduction of the AuNPs in the acidic medium was measured by differential pulse voltammetry (DPV) in a *KRAS* concentration-dependent manner. Likewise, Hua-Feng Wang et al. created a label-free ultrasensitive biosensor utilizing RNase III and terminal deoxynucleotidyl transferase for the detection of specific *KRAS* G12D mutation (Fig. 5a) (Wang et al., 2018). Similarly, electrochemical detection of *BRAF* V600E mutation was carried out by DPV following an amplification-refractory mutation system (Fig. 5b) (Situ et al., 2013). Thiolated and biotinylated amplicon was immobilized on Fe_3O_4/AuNP followed by loading alkaline phosphatases (ALPs) through biotin–streptavidin–ALPs interactions. The sandwich bioconjugate was magnetically attracted to the surface of the screen-printed carbon electrodes. The oxidation current of ascorbic acid was measured by DPV, which helped to detect *BRAF* V600E mutation in the colon cancer cells (HT29).

Electrochemical biosensors can detect cancer-associated epigenetic biomarkers as well. To determine the methylation pattern of the O^6-methylguanine-DNA methyltransferase (MGMT) gene, Povedano et al. (2020) developed the first electrochemical platform for an epigenetic biomarker (Fig. 5c). Their strategy involved the use of streptavidin-modified magnetic beads in conjunction with a biotinylated DNA capture probe. Subsequently, they performed hybridization with a synthetic DNA containing 5hmC in the promoter region of the *MGMT* gene. The electrochemical detection was done amperometrically using the H_2O_2/HQ system, which could detect the methylation of 5hmC in 10 ng of the genomic DNA extracted from colon cancer cell lines (SW480 and SW620) and the cancer tissues from CRC patients. For exosome analysis derived from cancer patients, an integrated magneto-electrochemical sensor (iMEX) was developed to profile the cell-derived exosomes (Jeong et al., 2016). After loading biotinylated antibody-conjugated magnetic beads together with the sample on a screen-printed electrode, the researchers sequentially performed chronoamperometric measurement, ELISA, and Flow Cytometry. Specific cells were then cultured, and the exosomes were harvested from there for downstream analyses. Likewise, Doldán et al. designed yet another electrochemical sandwich immunosensor to determine exosomes based on surface marker-mediated signal amplification using gold electrodes pre-functionalized with specific CD9 antibodies (Doldán et al., 2016). Moreover, a label-free electrochemical detection platform was developed that can identify human DNA (cytosine-5)-methyltransferase1 (*DNMT1*) in cultured colon cancer cells (HCT116) and colorectal tissue samples (Fig. 5d) (Furst et al., 2014). Another study developed a bioseparation and electrochemical detection method for exosomes using bioengineered and self-assembled superparamagnetic polyhydroxybutyrate (PHB) nanobeads (Soda et al., 2021). Functionalized PHB nanobeads with tetraspanin antibody CD9 facilitated bulk exosome capture. After magnetically capturing and purifying the exosomes, the exosome-bound antibody was immobilized on a CA125 antibody-modified electrode. After that, the electrochemical quantification of the CA125-expressing exosomes was done with the peroxidase activity of HRP through the hydrogen peroxide (H_2O_2)/HRP/hydroquinone (HQ) redox cycling system.

Apart from these, to determine the association of miR-21 with CRC, Zhou et al. designed a label-free electrochemical method using the hairpin structure probe and hemin-G–quadruplex complex (Zhou et al., 2012). A 5′-thiolated hairpin DNA probe S1 immobilized on a gold electrode was modified with electrodeposited AuNP, which opened the hairpin in the presence of the target miRNA, followed by hybridization. The 3′-end of the hairpin hybridized with a capture DNA S2 assembled on the surface of gold NPs along with the aptamer DNA S3. Lastly, the DNA S3 aptamer and hemin created the hemin-G–quadruplex complex, which represented the level of miR-21 quantified by an amperometric readout ranging from 5 to 5000 pM with a LOD of 3.96 pM. Lately, Boriachek et al. developed an amplification-free bioassay to detect exosomal miR-21 from the CRC cell line SW48 and the serum samples from eight CRC patients (Boriachek et al., 2018b). Boriachek et al. have also immobilized a biotinylated capture probe on the surface of streptavidin-labeled magnetic beads following hybridization of the target miRNA with the capture probe. The miR-21 was then isolated magnetically from the total exosomal RNA and was adsorbed onto the surface of a screen-printed gold electrode (SPAuE). The concentration of miR-21 was measured by DPV in the presence of $[Fe(CN)_6]^{4-/3-}$ redox system.

In addition to nucleic acid biomarkers, various proteins have also been the targets of interest for electrochemical assays (Ortega et al., 2023; Zhang et al., 2023). To detect carcinoembryonic antigen (CEA), Sales M.G.F.'s group designed a novel point-of-care electrochemical biosensor that is integrated into a Dye-Sensitized Solar Cell (Moreira et al., 2018). The device utilizes an interconnected photovoltaic cell as an independent energy source and a molecularly imprinted polymer that can recognize CEA with high affinity. Furthermore, a magnetic beads-based biosensor was prepared and altered with immobilized HaloTag fusion p53 protein, which acts as an electrochemical detection system (Garranzo-Asensio et al., 2016). The amperometric signal generated by the enzymatic reduction of H_2O_2 mediated by HQ following capturing the immunocomplex-containing magnetic beads onto screen-printed carbon electrodes revealed the level of p53 autoantibodies in the serum sample. Likewise, Islam et al. developed the first label-free electrochemical biosensor for quantitatively detecting the FAM134B protein as a biomarker for CRC (Islam et al., 2017c). A biotinylated anti-FAM134B Ab was attached at the surface of an extavidin-modified SPE, which was measured by DPV.

4.3. Optical methods

Surface plasmon resonance (SPR) entails tracking changes in refractive index by a thickened layer or mass accumulation on the sensor surface as a result of biomolecular surface interaction (Sina et al., 2016; Zhu et al., 2014). SPR provides the real-time and label-free readout of the target biomolecules since the change in refractive index is directly proportional to the change of mass on the sensor layer (Sina et al., 2016). Although SPR biomarker detection methods are now restricted to proof-of-concept research, it has the potential to be miniaturized and employed in a POC setting because of its promising detection approach and the cutting-edge manufacturing processes. Multiple biosensors based on SPR have been developed for the detection of CRC-associated biomarkers. For example, Springer and colleagues developed a biosensor with a fluidic SPR system for identifying the level of CEA, one of the most common biomarkers for CRC, in serum (Špringer and Homola, 2012). An anti-CEA antibody labeled on a gold chip surface via a carboxy-terminated linker was used as the sensing element. This simple biosensor had a LOD of 8 ng/ml, which was insufficient for CRC detection. Later, the same group of authors enhanced the analytical signal of the biosensor by applying functionalized gold nanoparticles (AuNP) (Ermini et al., 2019). This biosensor was able to identify CEA concentrations above 0.1 ng/ml. Another study by Wang and colleagues developed a dual signal amplification system for the detection of several proteins including CEA in clinical samples using AuNp-antibody and

antibody-quantum dot conjugates (Fig. 6a) (Wang et al., 2016a). The constructed SPR biosensor had an astounding detection limit as low as 0.1 ng/ml for CEA. Likewise, few other researchers employed similar strategies with SPR to detect the level of CEA in blood (Li et al., 2015a; Xu and Chen, 2018). More recently, Szymanska et al. developed a Surface Plasmon Resonance imaging (SPRi) immunosensor for determining plasma CEA level (Fig. 6b) (Szymanska et al., 2020). The immunosensor included a cysteamine linker attached to a gold chip and mouse anti-CEA antibody connected by the EDC/NHS protocol. This immunosensor had a linear response range between 0.40 and 20 ng/ml with a LOD of 0.12 ng/ml.

Alternatively, Surface-enhanced Raman scattering (SERS) is a highly sensitive vibrational spectroscopic technique that can enhance the Raman scattering by even a single molecule that is adsorbed on a rough nanometallic surface (Le Ru et al., 2006; Nie and Emory, 1997). This method can be employed to detect low-abundant biomolecules, typically in accordance with immunoassay-based approaches (Banaei et al., 2017). Several extensive literature reviews discussed the current state and future perspectives of SERS along with its diverse applications (Avram et al., 2020; Darroudi et al., 2023; Langer et al., 2020). Yulin Lu and colleagues recently developed a label-free molecularly imprinted polymers-SERS (MIP-SERS)-based biosensor to detect NDKB protein as a biomarker for CRC (Lu et al., 2023). Previously, Lin et al. identified that SERS intensity of many dominant vibrational bands appeared elevated in serum samples obtained from CRC patients compared to healthy individuals (Lin et al., 2011). Various other studies employed SERS for discriminating between cancer cells and healthy cells (Della Chiara et al., 2021; Li et al., 2015b; Lin et al., 2016; Tirinato et al., 2012). Also, Xiang et al. developed a SERS technique with Fe_3O_4/Au/Ag-based biosensor that could detect cytidine in the urine of individuals suspected of having CRC (Xiang et al., 2018). Processed urine mixed with Fe_3O_4/Au/Ag colloids was siphoned through a capillary tube followed by magnetic enrichment, separation, and Raman spectral experiments. Finally, liquid chromatography–mass spectrometry (LC–MS) was employed to evaluate the robustness of the Raman protocol. Furthermore, to address the limitation of detecting mutations from a long chain-dsDNA, Li et al. applied a PCR-SERS combination method (Li et al., 2018). This method incorporated the amplification strategy of PCR along with the detection features of SERS. They used this method for multiplex detection of *BRAF*, *KRAS*, and *PIK3CA* mutations in the plasma of CRC patients. Due to the narrow bands of the SERS, this combination PCR-SERS method is particularly convenient for simultaneous detection of multiple mutations. Another variant of this method involved combining asymmetric PCR with SERS which resulted in the highly specific detection of *KRAS* SNPs (Lyu et al., 2021). However, one drawback of these methods is that the mutated sequence needs to be

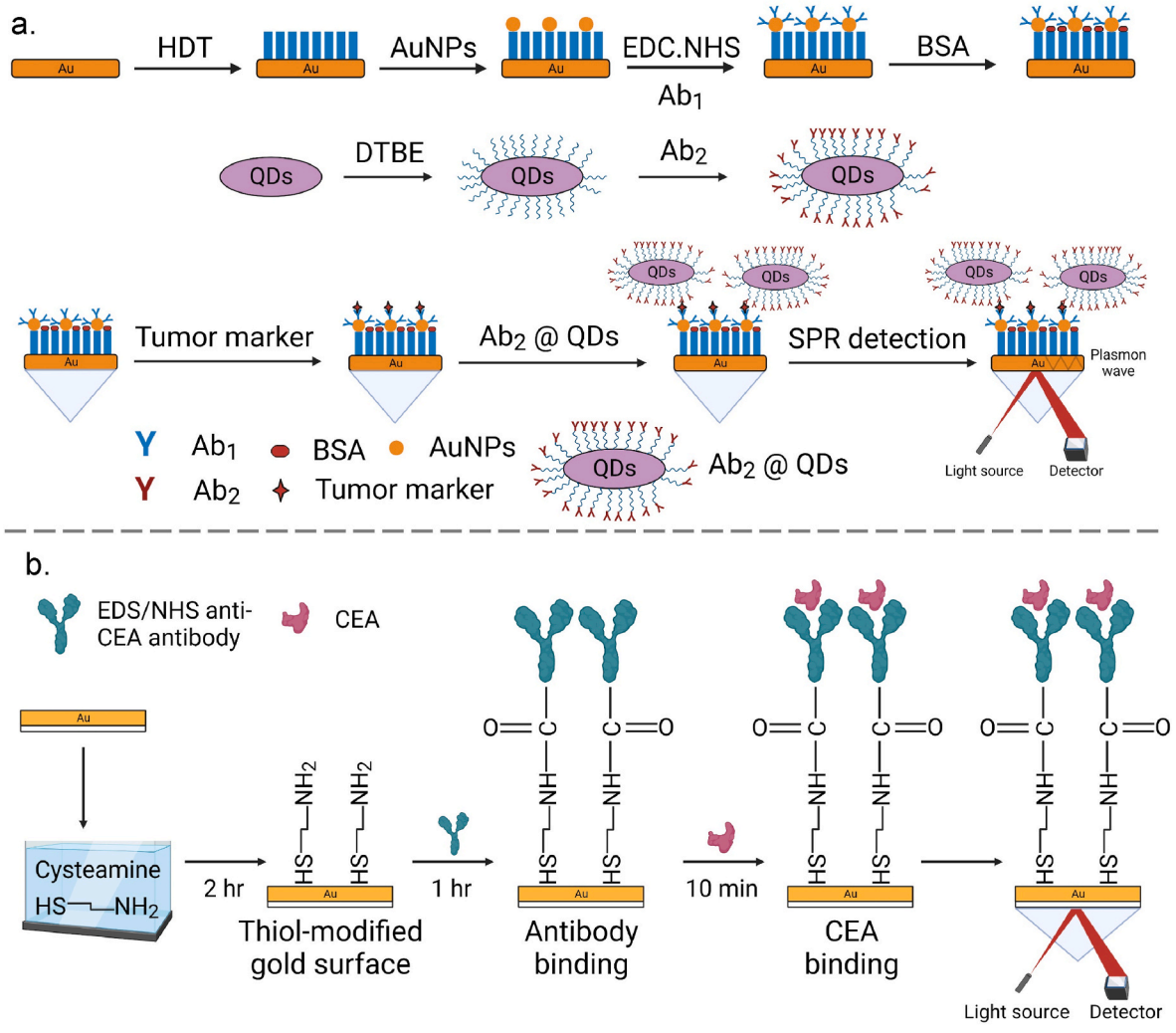

Fig. 6. Schematic representation of optical methods for biomarker detection. a. Tumor marker detection using an SPR biosensor coated with gold nanoparticles. Reproduced via Biorender under a Creative Commons Attribution License (CC-BY) from Ref (Wang et al., 2016a). copyright [2016] the authors. b. SPRi immunosensor using cysteamine linker for determining the level of CEA. Reproduced via Bioender from Ref (Szymanska et al., 2020). with permission from Elsevier, copyright [2020].

known beforehand for designing the probes (Li et al., 2018).

4.4. Nanoparticles

Different nanoparticles of varied sizes, shapes, and compositions have become potential tools for CRC diagnosis, staging, and treatment (Djermane et al., 2023; Dong et al., 2016). Wang et al. used surface plasmon resonance (SPR) based on stable graphene oxide gold NPs (GO-AuNPs) hybrids to detect miRNAs from CRC (Wang et al., 2016c). They immobilized the thiolated DNA probe with a short complementary sequence on the surface of gold film and captured part of the target miRNA. The other part of the target was attached to the GO-AuNPs hybrid. The resulting electrode could distinguish between different members of the miRNA-200 family and detect highly sensitive human miRNAs. The resulting electrode could distinguish between different members of the miRNA-200 family and detect highly sensitive human miRNAs. Several magnetic and AuNP-based approaches have been utilized for the detection and isolation of circulating tumor cells (CTCs) in cancer patients. Some of these approaches include tannic acid-functionalized magnetic NPs (Ding et al., 2021), peptide-based magnetic NPs (Jia et al., 2021), $CoFe_2O_4$@Ag magnetic NPs (Vajhadin et al., 2022), two-dimensional nanozymes with AuNPs (Yang et al., 2021), and Ab-functional microspheres integrated filter chip (Su et al., 2019).

Several studies involved quantum dots (QDs) (also called luminescent semiconductor nanocrystals) such as ZnS, CdS, CdSe, CdTe, and PbSe for the detection of cancer biomarkers (Pericleous et al., 2012). Factors such as high sensitivity, stable fluorescence with simple excitation, multispectral tunability, resistance to photo bleaching, and no requirement for lasers make QDs promising candidates for tumor biomarker detection (Brar et al., 2021). In one study, Zhang et al. synthesized microfluidic bead-based nucleic acid sensors tagged with QDs that could detect fragments of *CEA* with a LOD as low as 5 fM(Zhang et al., 2013). It also reported the detection of CTCs effectively, which could distinguish a single cancer cell in one ml of blood. In another study, Wang et al. used a QD-based immunohistochemistry (QD-IHC) method and analyzed the expression level of large external antigens in the cancer tissue samples obtained from patients with CRC (Wang et al., 2016b). Detectable advantages of QD-IHC included less human interference, higher sensitivity, and better capacity for simultaneous multifactor analysis. Thus, this method can be used in therapeutic applications.

These showed promising results in terms of sensitivity, high capture efficiency, inhibiting nonspecific adhesion of peripheral blood mononuclear cells, and cost-effectiveness. However, current knowledge of pharmacokinetics, biodistribution, metabolism, and nanoparticle clearance is still lacking due to their shortage of clinical trials. Furthermore, there remain potential cytotoxic issues with nanoparticles due to the accumulation of heavy metals in the organs and interaction with the biomolecules, cells, organs, and tissues (Brar et al., 2021).

Table 3 summarizes the major advantages and limitations of the advanced analytical methods for the detection of biomarkers associate with CRC.

Table 3
Summary of advanced analytical detection methods for CRC.

Method	Advantages	Limitations	Ref.
Microfluidics	Efficient cell sorting, quantification and single-cell analysis. Minimal sample preparation required. Small amount of sample required. Multiplexing capability. Reduced cost for analysis. High throughput.	Complex and expensive equipment. Complex fabrication process. Variability among devices. Potential clogging of microfluidic channels. Captured cells cannot be used for further downstream analyses. Elevated false positive results from non-specific interactions. Technical issues in handling small sample volumes.	(Akgönüllü et al., 2021; Bargahi et al., 2022; Chin et al., 2012)
Electrochemical biosensors	Simple and inexpensive. High sensitivity and specificity. Minimal sample preparation required. Easy to fabricate and integrate with existing equipment.	Poor stability of bio-recognition elements. Possible interference from background signals. Potential for oxidation of the electrodes. Limited multiplexing capability. Limited storage stability.	(Hasan et al., 2021; Topkaya et al., 2016; Xu et al., 2018)
SPR	High sensitivity and specificity. Real-time monitoring of binding events. Ability to multiplex various samples. Label-free analysis.	Expensive. Complex sample preparation. Need for highly skilled personnel to operate and interpret the results. Limited availability of specific biosensors for each biomarker.	(Bellassai et al., 2019)
SERS	High sensitivity and specificity. Label-free detection. Compatible with a variety of samples. Non-destructive analysis. Ability to analyze both organic and inorganic molecules.	Expensive. Complex sample preparation. Need for highly skilled personnel to operate and interpret the results. Possible interference from bacground species in the sample. Low reproducibility due to variability in surface enhancements. Limited spatial resolution.	(Langer et al., 2020; Vendrell et al., 2013)
Quantum dots	High sensitivity and specificity. Bright and stable fluorescence emission. Versatile labeling ability for different types of biomolecules. Long-term stability and shelf life.	Expensive. Potential for toxicity due to the use of heavy metals. Complex synthesis and purification process.	(Devi et al., 2022; Zhang et al., 2008)

(*continued on next page*)

5. Challenges associated with biomarker detection

The major drawback of detecting CRC-associated biomarkers is the lack of a consistent and specific method. For earlier detection of CRC, an efficient and reliable diagnostic method with acceptable sensitivity and accuracy is required. Various challenges associated with biomarker detection are discussed below.

5.1. Biological challenges

Several biological parameters, including sample heterogeneity, low abundance of biomolecules, and varied fragmentation patterns, pose

Table 3 (*continued*)

Method	Advantages	Limitations	Ref.
Nanoparticles	High sensitivity and specificity. High surface area-to-volume ratio provides large binding capacity for biomarkers. Ability to target specific cell types. Multiplexing ability. Versatility for functionalization with different targeting moieties.	Complex synthesis and functionalization methods. Potential toxicity and biocompatibility issues. Difficulty in controlling size and shape of nanoparticles. Limited commercial availability and high cost.	(Yao et al., 2020)

Abbreviation: SPR – surface plasmon resonance; SERS – surface-enhanced Raman spectroscopy.

significant challenges in detecting biomarkers from patients with CRC. These challenges will be addressed in the following section.

5.1.1. Sample heterogeneity

Different genetic, physiological, and environmental factors related to sample heterogeneity may affect the detection of the biomarkers. Some of the biomarkers (i.e., CTCs, exosomes) may be present irregularly even in a healthy individual, making it difficult to compare with the diseased condition. Moreover, the presence of heterozygous alleles, aneuploidy, SNPs and/or translocations in the genes responsible for CRC pathogenesis may lead to diminished or altered detection levels. This can be attributed to the difference in age, gender, body mass index (BMI), and immune microenvironment varying from person to person (Witwer et al., 2013). Thus, choosing an ideal control for a greater cohort of heterogeneous sample is a significant challenge. Regular experiments are needed to evaluate the effects of sample heterogeneity on the quantity, development, and functions of the biomarkers. Creating a sample control cohort consisting of every possible variant based on age, gender, race, and physiological conditions can potentially overcome this challenge as well.

5.1.2. Low abundance

Although blood samples withdrawn from the veins should represent the whole blood of the body, they may not reflect the real population for some biomarkers such as CTCs and ctDNA. These markers are present in a shallow frequency compared to the high background of non-neoplastic blood cells and cfDNA (Yu et al., 2011). The significantly lower concentration of these biomarkers makes their detection, characterization, and analysis challenging. This often leads to false negative identification of cancer cells. This can be circumvented by either increasing the sample volume within the clinically allowable range and/or introducing an enrichment step before detection.

5.1.3. High fragmentation

The high fragmentation of circulating nucleic acids poses a challenge in their isolation, as it impacts the accuracy of quantitation (Sedlackova et al., 2013). Fragmentation of DNAs considerably decreases the efficiency of amplification and sequencing techniques (Gorgannezhad et al., 2018). Additionally, the variable fragment size of ctDNAs adds to the complexity. In healthy individuals, cfDNAs typically consist of smaller fragments (~185–200 bp) due to apoptosis. On the other hand, the ctDNAs with variable fragment lengths originated from solid tumors through necrosis and autophagy due to incomplete DNA digestion (Ivanov et al., 2019; Jung et al., 2010). This ultimately may jeopardize the accuracy of ctDNA analysis due to the potential loss of target sequence.

5.1.4. Difficulty in direct analysis

Typically, amplification, sequencing, and hybridization-based approaches are employed for the analysis of genetic biomarkers. Although hybridization confirms sequence specificity, this involves laborious and time-consuming steps (Noh et al., 2015). Frequent denaturation of the dsDNA followed by renaturation hinders the efficiency of the hybridization-based analyses (Noh et al., 2015). Further studies on electrochemical detection of dsDNA using alkaline phosphatase-labeled Zn-finger proteins and DNA clutch probes (DCPs) enable efficient hybridization analysis (Das et al., 2016; Noh et al., 2015).

5.1.5. Low stability

Due to the short half-life of various biomarkers (e.g., 4–30 min for cfDNA), isolating and detecting them quickly poses a challenge (Lo et al., 1999). Analyzing RNAs is also challenging due to the abundance of RNases in the environment. The presence of diverse nuclease activities and varying environmental conditions can alter the stability of genetic biomarkers, thereby impacting diagnostic strategies and often yielding inconsistent and insufficient results. Furthermore, certain biomolecules, particularly exosomal miRNAs, are shielded from degradation (e.g., by RNases) through encapsulation within a protective microenvironment. This necessitates additional complex steps during downstream analysis. The detection of biomarkers can be hindered by factors such as intratumor heterogeneity, low tumor cellularity, and the presence of adjuvant chemotherapy (Li et al., 2017c). Prolonged incubation of tissue or blood samples can also result in inaccurate biomarker profiling due to nonspecific alterations.

5.2. Technical challenges

In addition to biological challenges, there are several technical aspects that need to be addressed when detecting CRC-associated biomarkers. These aspects will be discussed in the following section.

5.2.1. Sample source and preparation

Several preanalytical factors such as sample collection, sample processing, the time interval between collection of sample and centrifugation, storage conditions, centrifugation force, and cryopreservation conditions can induce altered levels of the biomarkers (Sozzi et al., 2005). The diagnostic platforms need to be standardized following regular calibration to overcome this issue. To date, numerous methods have been established to investigate the biomarkers obtained from CRC patients. One of the major limitations of these technologies is lacking a gold standard isolation strategy. For instance, to analyze the biomarkers (i.e., DNA/RNA/protein) from tissue samples, several different extraction methods with various steps and reagents need to be employed. Each of these methods may result in varying amounts and quality of the biomarkers.

Furthermore, in the case of circulating biomarkers such as exosomes, cfDNA, CTCs, etc., no specific isolation method is also prescribed. The most used method for exosome isolation from body fluids is ultracentrifugation due to their size and density. However, ultracentrifugation is time-consuming, expensive and can be laborious. The exosomes are also exposed to huge pressure due to ultracentrifugation, and the specificity during precipitation is less (Li et al., 2017b). Additionally, reproducible isolation is quite challenging in the case of varied settings or places.

On the contrary, while ultrafiltration is relatively faster and requires no specialized equipment, the high force may lead to deformed and broken vesicles (Batrakova and Kim, 2015). In the case of CTC isolation, both positive enrichment of EpCAM-expressed CTCs and negatively selecting WBCs by depleting CD45-expressing cells can be employed (Allard et al., 2004; Kang et al., 2019). Various commercial kits are now available for extracting and purifying these biomarkers efficiently, which are faster and reproducible, albeit expensive.

Another frequent challenge during sample collection is the infiltration of the impurities generated by activated platelet-derived vesicles

due to physical forces while drawing blood. Moreover, contamination of the samples during isolation, preservation, or detection of the biomarkers severely enhances inaccuracy. A standardized sampling size is needed to overcome shearing stress and, thereby, platelet activation. Larger needles can be used while carefully drawing blood to avoid platelet activation (Witwer et al., 2013). Additionally, recent studies reported that heparin prevents the recipient cells to uptake extracellular vesicles, thus, it is suggested to avoid using heparin-based anticoagulants (Maguire et al., 2012). Alternatively, ethylenediaminetetraacetic acid (EDTA), sodium fluoride, or sodium citrate can be used in the blood collection tubes. Detection of small amounts of biomolecules in the presence of high impurities is another challenge with the sample preparation. This requires a highly sensitive and specific diagnostic method to avoid false negative results.

5.2.2. Low efficiency of extraction procedures

Due to sample heterogeneity, various biomarkers (i.e., CTCs) display varied phenotypes, which calls for a comprehensive enrichment spectrum using specific cell surface markers (Yu et al., 2013). However, combining all different markers simultaneously may lead to false positive results. To circumvent this issue, actin bundling protein plastin-3 has been targeted, which is not expressed by the blood cells, while not downregulated by CTCs either. A study by Praharaj et al. developed a microfluidic CTC-iChip that can differentiate between epithelial and non-epithelial cells using either a label-dependent or a label-free approach (Praharaj et al., 2018). Furthermore, technologies such as flow cytometry and laser capture microdissection (LCM) can also be implemented for additional isolation of CTCs (Zhu et al., 2018).

Despite the advances in cfDNA/ctDNA isolation and purification strategies, a significant amount of them is still lost during purification steps. Advancements in microfluidics and nanotechnology brought forth new cfDNA isolation techniques with better yields (Sonnenberg et al., 2014). Likewise, nanochip and nanowire-based methods have also enhanced the recovery of cfDNA from plasma, followed by their release by switching the oxidation state of the conducting polymer (Lee et al., 2016). Microfabrication techniques have rendered time-efficient ways for isolating cfDNA, as evidenced by Campos et al., who developed a microfluidic device that can efficiently extract cfDNA directly from plasma, thus enhancing the overall sensitivity of ctDNA detection strategies (Campos et al., 2018).

5.2.3. Limited comparability of various quantification methods

Over the years, many quantification methods based on spectrophotometry and fluorometry have been developed that utilize diverse types of dyes and PCR-based amplification techniques targeting various nucleic acid biomarkers (DNA/RNA/cDNA/ctDNA) (de Kok et al., 1998; Lee et al., 2018). A typical qPCR provides numerous copies of a single DNA fragment, on the contrary, an isothermal amplification like LAMP provides a concatemer of the amplified target DNA (Notomi et al., 2000). Moreover, the fluorometric approach targets total cfDNA levels without any DNA length limitation, whereas PCR-based techniques need shorter DNA to allow ctDNA detection (Devonshire et al., 2014; Horlitz et al., 2009). As an optimum strategy for the efficient and definitive detection and quantification of the biomarkers is still lacking, a general standard operating procedure and standardized approach is required considering all the pre-analytic issues that may hamper the test results.

5.3. Clinical challenges

Determining the biomarkers related to CRC requires large-scale clinical studies and sufficient evidence for proving the efficiency of the diagnostic assay, reliability of the biomarker, and clinical feasibility of the developed method. To effectively confirm the association of biomarkers, it is crucial to analyze large cohorts of both CRC patients and healthy individuals as controls. Continuous clinical follow-up of these cohorts is also necessary to distinguish between true and false positive results (Genovese et al., 2014). Furthermore, conducting multiple experiments and validating the findings with alternative methods are essential steps to obtain conclusive results with clinical samples.

6. Which biomarker and detection method are most widely used and why?

So far, in this review, we have addressed the commonly used biomarkers derived from CRC as well as the conventional and advanced methods for their detection along with the associated challenges. Among all these diverse biomarkers, *KRAS* mutation status testing is the most widely used in the clinical setting for cancer prognosis. The primary factor for this is that the presence of a *KRAS* mutation is linked to the rapid aggression of cancer and can aid in evaluating how patients with metastatic CRC respond to treatment. *KRAS* oncogene is mutated in approximately 35%–50% of CRC patients (Dai et al., 2020; Formica et al., 2022). Almost 85% of *KRAS* mutations include one of the three mutation hotspots in codons 12, 13, and 61, which were confirmed as negative predictors of response against anti-EGFR antibodies (Haigis, 2017). Among these three, mutations in codon 12 are predominant (65%) with G12V and G12D mutations being the most common subtypes in CRC. Thus, determining the *KRAS* mutational status of either tumor or blood samples has become an integral tool in predicting treatment response in patients with CRC. However, detecting the mutational status of *KRAS* alone is not sufficient and additional tests are required in non-responsive patients.

As discussed above, genetic mutation is the most widely used biomarker for CRC. For mutation detection, qPCR followed by direct sequencing is still considered as the gold standard method. qPCR allows for precise real-time quantification of target DNA sequences, while direct sequencing can provide highly accurate readout of these sequences. These two methods in conjunction facilitate the detection of mutations when compared to the reference sequence. Moreover, these techniques are also highly versatile and can be used to analyze DNA from various sources, including blood, tissue, and saliva.

7. Conclusion and future perspective

Over the years, liquid biopsy, in contrast to conventional tissue biopsy, has become a game-changing tool in precision medicine management for cancer. The potential of circulating biomarkers, such as CTCs, cfDNA/ctDNA, and exosomes in translational cancer research is now well appreciated and thus implemented in various clinical trials. Consequently, diverse molecular techniques have come forth to detect these biomarkers. However, multiple inherent limitations of these detection methods need to be addressed to evaluate their roles in clinical settings. This review aimed to provide an overview of the common biomarkers of CRC and the current state of the methods for detecting these biomarkers, along with their methodological disadvantages.

Traditional amplification and gene sequencing is widely used for the detection of CRC-associated biomarkers, however, most of these methods lack sufficient sensitivity as well as require significant time and resources. For instance, PCR-based strategies are predisposed to some error rates for DNA polymerase that limits the application of PCR-based techniques in amplification-sensitive assays. Furthermore, improved, and sensitive diagnostic assays are needed due to the lack of recurrent alterations in cfDNA/ctDNA population. Although several attempts have been employed to develop reliable assays for analyzing the tumor genome derived from ctDNAs, most of these procedures are tedious and costly, limiting their application in the clinical setting.

The lack of techniques for effectively isolating and detecting CTCs renders a significant challenge to understanding CTC biomarkers in clinical settings. Using diverse biomarkers for the capturing and/or enrichment of CTCs is a notable limitation of CTCs. High throughput analytical techniques like NGS can be employed to analyze the whole genome and transcriptome of single CTCs, that can significantly increase

the molecular and functional characterization of CTCs. WGA of the single CTCs following the amplification of specific genetic biomarkers can also be an effective strategy.

More importantly, in recent times, CRISPR/Cas-based methods have been utilized for various cancers, including breast cancer (Deng et al., 2021), non-small cell lung cancer (Jia et al., 2018; Liu et al., 2022; Wang et al., 2023b), as well as in cancer cell lines (Song et al., 2021; Tsou et al., 2020; Xiao et al., 2020; Zhou et al., 2022), and synthetic DNA (Pang et al., 2022; Wang et al., 2023a) for the identification of single base mutations. There has been no specific research conducted solely on CRC using the CRISPR/Cas system to date. However, these studies have clearly demonstrated that the CRISPR/Cas system have potential to be employed in determining the mutation status of genes responsible for CRC.

While no single biomarker is sufficient for the diagnosis and prognosis of CRC, targeting multiple biomarkers simultaneously may prove to be a more effective approach. Some of these technologies have the potential for multiplexing and can be performed in point-of-care (POC) settings. Moreover, nanotechnology based POC testing offer rapid and cost-effective results while maintaining high sensitivity for the target biomarkers. Novel strategies such as PCR clamping by peptide nucleic acid (PNA) and locked nucleic acid (LNA), ligase chain reaction (LCR), CRISPR/Cas-based techniques, ingestible electronics, and the conjugation of nanobodies to nanoparticles can be implemented for the early detection of CRC biomarkers, potentially in a POC setting.

Author contributions

M. S. Islam completed the literature survey, wrote, and edited the manuscript. M. J. A. Shiddiky, V. Gopalan, and A. K. Lam reviewed and edited the manuscript. M. J. A. Shiddiky, and V. Gopalan were involved in conceptualization, and administration of the project. All authors approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2023.115611.

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