Khalid El Bairi *Editor*

# Illuminating Colorectal Cancer Genomics by Next-Generation Sequencing

## A Big Chapter in the Tale

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*We wrote this book with love for oncology and cancer science.*

*Khalid El Bairi*

*To my parents (Mohammed and Fatima) and my family for always supporting me*

*To Dr. Hind Kadiri (PhD) and Pr. Maryam Fourtassi (MD, PhD)*

*And particularly to cancer patients*

# Preface

Colorectal cancer is a devastating disease and is the second most common cause of cancer death annually worldwide. Although screening programs have reduced deaths from colon cancer in older age groups, worryingly and for unknown reasons, the incidence of colorectal cancer in younger populations is increasing rapidly. The management of colon cancer has changed fundamentally over the past decade, moving from generic chemotherapy regimens to a more personalized approach. Cancer genomics has been at the heart of these changes; for example, *RAS* testing has improved patient selection for anti-EGFR therapy, and *BRAF*-directed treatment has emerged as a new standard in $BRAF^{V600E}$ mutant colorectal cancers.

It is a great pleasure to be asked to write a preface to this book. The initial chapters build on a review of the underlying genetic molecular changes found in colon cancer and extend to the rationale for personalized therapies. Potentially transformative technologies including liquid biopsies and single-cell sequencing are discussed in detail. An important debate around the cost and value of next-generation sequencing in colorectal rounds off the text. This manuscript will be enjoyed by all those who are interested in furthering their knowledge on the genomics of colorectal cancer using next-generation sequencing.

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Elizabeth C. Smyth

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# 1 An Introduction to the Current Management of Colorectal Cancer in the Era of Personalized Oncology

Angelica Petrillo, Emanuela Ferraro, Michele Ghidini, and Dario Trapani

**Abstract**

Until recently, disease indications for anticancer drugs have typically been based on histological findings and cancer staging. Remarkably, several predictive biomarkers have been recently added to conventional schemes to select patients who may be more likely to benefit from treatments. In colorectal cancer, fluoropyrimidine-based chemotherapy is still the backbone of systemic treatment. Other drugs such as irinotecan and oxaliplatin as well as emerging targeted agents combined with 5-fluorouracil have significantly improved survival rates. Moreover, the advent of precision oncology procedures has enabled better decision-making algorithms particularly with implementation of molecular pathology and targeted anticancer agents, including immune-checkpoint inhibitors that may radically change the management of this disease and its outcomes in the coming years.

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**Keywords**

Colorectal cancer · Therapy · Biomarkers · Precision medicine

## 1.1 Introduction: A Brief Overview of the Implications of Translational Medicine to Inform the Clinical Decision-Making in Colorectal Cancer

Colon and rectal cancer (CRC) represents the third most diagnosed and the second cause of death from cancer in the world, accounting for 1.8 million new cases and nearly 900,000 related deaths in 2018 (Bray et al. 2018). The traditional classification of colorectal malignancies takes into account anatomic and histology factors, including the localization, the sidedness, the histology differentiation, and the locoregional and distant invasiveness (Bosman et al. 2010). However, more recently, the traditional paradigm based on the histology classification has been refined by more sophisticated characterizations based on molecular genomics. In 2015, Guinney et al. reported the first consensus molecular subtypes of CRC, aiming to develop a framework for the definition of intrinsic subtypes of this disease (Guinney et al. 2015). The report identified four consensus molecular subtypes (CMSs) with distinguishing features and biological behaviors: (1) CMS1 or microsatellite instability immune, exerting strong immune activation; (2) CMS2 or epithelial/canonical, showing mainly WNT and MYC signaling activation; (3) CMS3 characterized by metabolic dysregulation; and (4) CMS4 with mesenchymal phenotype, in which high transforming growth factor-beta (TGF-β) activation, stromal invasion, and angiogenesis are described (Guinney et al. 2015). Conversion to CMS4 may occur as a result of a selective resistance pressure to chemotherapy (CT) and other agents (Guinney et al. 2015). In the first validation of the consensus, a clinic and prognostic significance was suggested for the intrinsic subtypes. CMS1 tumors were shown to be more common in females, presenting as high-grade tumors of the right colon. On the contrary, the CMS2 subtype is prevalent in the left-sided CRC. In contrast, mesenchymal types are detected more commonly as locally advanced or metastatic, exhibiting an intrinsic poorer prognosis. The recognition of the intrinsic subtypes has allowed tailoring CRC beyond a mere consideration of histology subtypes, addressing selected mutations or reproducible patterns of alterations. To date, these major advances are not yet included in the practice guidelines of CRC management. However, other notable achievements in therapeutics and biomarkers are now considered in CRC major guidelines (e.g., NCCN and ESMO), including targeted agents and immune-checkpoint blockade.

Although landmark trials clearly delineated the field of application of adjuvant treatment in CRC, nowadays it is impossible to anticipate the benefit of treatments in this setting, and few molecular biomarkers have been proposed in clinical practice. Based on this assumption, the research about the factors that can help to predict the response to treatment at the time of diagnosis became important in order to assign patients to the most appropriate tailored treatment. In this context, the molecular

consensus previously reported represents the first step toward a new precision medicine vision in the CRC field (Guinney et al. 2015). In fact, understanding the molecular alterations that are behind the clinical behavior of a subgroup of tumor might represent the base for the development of new therapeutic strategies. However, the consensus molecular classification has not been yet validated in adjuvant setting, and only a few alterations are targetable in the context of metastatic disease. Therefore, the consensus requires further prospective validations in order to be useful to tailor the treatment for the right patient in the correct timing.

Several multigenic assays as well as pathological, biological, and molecular factors have been investigated over the last few decades in order to overcome this gap. Among these factors, the determination of microsatellite and mismatch repair protein status (MMR) has an important prognostic and predictive role. Mutations of MMR genes could lead to MMR deficiency (dMMR) or microsatellite instability [MSI, divided into MSI-high (MSI-H) or low (MSI-L) depending on the significant presence or not of instability]. MSI-H is found in patients with Lynch syndrome (2–4%) (Beamer et al. 2012) or in sporadic cases due to somatic alterations in about 19% of CRC, more frequent in stage II tumors than in the metastatic one (3.5%) (Roth et al. 2010). Patients carrying stage II colon cancer with MSI-H and/or dMMR showed a better prognosis with low risk of recurrence and less benefit by using adjuvant CT based on fluoropyrimidine agents (Sargent et al. 2010). The biological explanation of this association might be the high tumor mutational burden (TMB) owing to dMMR. High TMB can enhance the generation of effective neoantigens, which may lead to the activation of cytotoxic T cells forming the tumor-infiltrating lymphocyte (TILs), capable of antitumor immune response. Thus, MSI-H patients have generally better prognosis. Regarding the predictive meaning of MSI, most studies comparing the effects of fluorouracil-based CT versus (vs.) observation revealed no significant improvements in overall survival (OS) or disease-free survival (DFS) in patients with MSI-H/dMMR tumors. On the other hand, patients with MSS or MMR proficient colon cancers benefit from the treatment. Based on these results, the determination of MMR status became part of daily clinical practice in patients with stage II CRC before starting adjuvant CT as well as in patients with a familial history of Lynch syndrome, according to more recent international guidelines (Schmoll et al. 2012). In patients with stage III disease, the risk of recurrence is higher, and therefore, they should receive standard CT regardless of MSI status. The main multigenic assays used are the Oncotype Dx Colon Cancer, the ColoPrint, and ColDx. All these assays analyze a pattern of genes that might be related with recurrence in order to create a score able to predict the prognosis for patients with stage II and III CRC (Jiang et al. 2008; Salazar et al. 2011). However, at the moment, there is insufficient evidence to support their use as prognostic-predictive tools in the clinical practice, and therefore, further studies are required for validation.

Another type of scoring—the immunoscore—was developed to give a prognostic meaning to some immunological features, such as TILs. Recently, a trial involving 1130 patients with stage II naïve CRC showed that patients with high immunoscore, considered high-risk patients, had no different time to recurrence compared to low

risk (Galon et al. 2019). Caudal-type homeobox transcription factor 2 (CDX2) is another promising prognostic and predictive factor investigated in the setting of early colon cancer. CDX2 is a regulator of the embryonic development of the gut and might have a role in oncogenesis; its expression is highly specific for the intestinal epithelium (Beck and Stringer 2010; Chawengsaksophak et al. 1997). Based on this assumption, CRCs without CDX2 expression (4.1–6.9% of CRC) are often associated with aggressive features such as advanced stage, poor differentiation, vascular invasion, *BRAF* mutation, and the CpG island methylator phenotype (CIMP). A recent trial showed that, while the lack of CDX2 expression was associated with a worse outcome in stage II and III CRC, adjuvant treatment added benefit in both CDX2-positive and negative tumors in stage III due to the high risk of relapse related to lymph node involvement. On the other hand, in stage II tumors, where adjuvant CT is administered only in the presence of high risk of recurrence, CDX2 might select a subgroup of patients that have higher risk and could benefit from adjuvant treatment (Dalerba et al. 2016). However, CDX2 test is not a standard of care in clinical practice today, especially for the cost and for the low prevalence of positivity in the population (for instance, the screening of 100 patients is required to find 4 cases of lack of CDX2). Moreover, recent research has been focused on *PI3KCA* mutations, which is detected in 10–20% of CRC. Patients with *PI3KCA* mutation seem to benefit from adding aspirin to adjuvant treatment, especially in stage III colon cancer, showing better DFS and OS (Ng et al. 2015). However, also in this case, these findings need to be validated in further trials.

One of the first attempts to investigate the role of circulating tumor DNA (ctDNA) and liquid biopsy in CRC was just done both in the early and metastatic disease. Regarding the adjuvant setting, a large prospective trial evaluated ctDNA to detect minimal residual disease in 1046 plasma samples of 230 patients with resected stage II colon cancer. The trial showed that ctDNA detection after surgery could discriminate patients with a substantial increase of the risk of recurrence. In particular, in patients never exposed to adjuvant CT, ctDNA was detected postoperatively in 7.9% of cases. Those patients showed a disease relapse in 79% of cases after a median follow-up of 27 months. On the contrary, recurrence occurred in only 9.8% of patients with negative ctDNA (HR: 18; 95% CI: 7.9–40; $p < 0.001$) as well as the presence of ctDNA after adjuvant CT predicted better outcomes in terms of recurrence-free survival (RFS) rate (Tie et al. 2016). In summary, tumor stage, tumor grade, and MSI remain the most important validated prognostic factors that might guide the choice of treatment of patients with early-stage colon tumors. At this time, there is no direct relation between the intrinsic molecular subgroup of CRC and the adjuvant treatment. Prognostic variables such as lymphovascular or perineural invasion, immunological biomarkers, or ctDNA might be very promising in the future. All these findings emphasize the opportunities to identify predictive markers able to guide the decisions for adjuvant CT in daily clinical practice. In this chapter, the current advances in the therapeutic management of CRC in the era of precision medicine are discussed. Additional details about predictive biomarkers of response to systemic therapy in CRC such as *KRAS* and *BRAF* are discussed in detail in

Chaps. 2 and 4. Here, we give an overview of the current treatments of CRC in the context of precision oncology.

## 1.2 Neoadjuvant Therapy

Historically, surgery is the milestone for curative treatment of operable patients with locally advanced rectal cancer (LARC). The improvement of surgical techniques with the introduction of total mesorectal excision (TME) and the addition of neoadjuvant chemo-radiation treatment (NA-CRT) before surgery have significantly reduced the 5-year local recurrence from >25% to approximately 5–10% for LARC (Swedish Rectal Cancer Trial et al. 1997; Sauer et al. 2004; Bosset et al. 2006). The NA-CRT has been validated in several studies compared to postoperative chemo-radiation treatment (CRT). The study published by the German Rectal Cancer group showed a lower rate of 5- and 10-year recurrence rate (6 vs. 13%, $p = 0.006$ and 7 vs. 10%, $p = 0.048$, respectively) and a better toxicity profile, with no differences in terms of DFS and OS between the two arms (Sauer et al. 2004). The standard NA-CRT is characterized by CT with 5-fluorouracil (5-FU)/capecitabine as radiosensitizers administered concomitantly to long-course radiation (50.4 Gy in 28 fractions) followed 4–8 weeks later by surgery. Alternatively, short-course radiotherapy (RT) at 25 Gy in 5 fractions could be an option in selective cases in which lower radiation toxicity should be guaranteed. Indeed, no significant difference in terms of local recurrence has been shown in Polish and TROG studies (the most relevant trial in this field) compared with conventional long-course radiation (Bujko et al. 2006; Ngan et al. 2012). More recently, the optimal timing to surgery after the short-course RT (1 week or 4–8 weeks) compared to the standard long-course RT (followed by 4–8 weeks to surgery) was investigated in a controlled randomized phase III clinical trial (Stockholm III). Overall, the data support a delay of 4–8 weeks to surgery, after short-course RT, as associated with a lower risk of postoperative complications (Erlandsson et al. 2017).

Other agents have been investigated as radiosensitizers in NA-CRT in order to potentiate the pathological complete response (pCR) rate. The combination of oxaliplatin with 5-FU failed to improve the response rate and to reduce the percentage of sphincter preservation surgery but had more overall toxicities including grade 3–4 diarrhea (Allegra et al. 2015). Irinotecan seems to be promising in combination with capecitabine as shown in phase II RTOG study (Wong et al. 2012). The efficacy and safety in high-risk LARC are being evaluated in the ongoing ARISTOTLE trial ( ISRCTN09351447). Another ongoing phase III trial, CinClare study (NCT02605265), is designed to demonstrate the superiority of the combination of weekly irinotecan and 5-FU, establishing the dosage of irinotecan according to the UGT1A1 genotype, a gene involved in the metabolism and, therefore, adjusted for the possible toxicity and bioavailability of the camptothecin derivative. Molecular targeted agents represent another alternative for addition to 5-FU-based regimen. Considering the role in *KRAS* wild-type (wt) advanced CRC, several phase I and II studies including epidermal growth factor (EGFR) inhibitors, cetuximab and

panitumumab, have been performed, failing to demonstrate a significant additional benefit. In the NEORIT trial, panitumumab was combined with standard NA-CRT protocol for a selected population of *KRAS*$^{wt}$ LARC. This combination therapy showed a favorable toxicity profile and did not appear to compromise surgical morbidity. Even if the downstaging of primary tumor was observed in 65% of the cases, the pathological complete response (pCR) rate was achieved in only 3.7% of the population vs. an expected $\geq$15% (Merx et al. 2017). Panitumumab has been investigated also as single agent administered in combination with long-course RT in low-risk LARC (mid-low rectum, cT3N− or cT2–T3N+, *KRAS*$^{wt}$ status, and negative circumferential radial margin) in the phase II RaP/STAR-03 trial, which did not meet the primary endpoint of pCR (Pinto et al. 2018). Similar negative results have been obtained with trials investigating cetuximab (Eisterer et al. 2014) in addition to standard concomitant CT with fluoropyrimidine-based regimen +/− oxaliplatin (Rodel et al. 2008) or irinotecan (Hong et al. 2007; Horisberger et al. 2009). The biological mechanisms related to these negative results of the studies may be related to the cetuximab-induced arrest of tumor cell cycle in phase G1 that makes cells less sensitive to CRT (Narvi et al. 2018), thus impairing the efficacy of concomitant tumoricidal treatments.

Additionally, trials investigated the combination of antiangiogenic agents (antivascular endothelial growth factor (VEGF)) with NA-CRT, showing that the inhibition of the VEGF pathway can enhance radiosensitivity, by inhibiting the neoangiogenesis and reducing the vascular density for tumor-associated endothelial cells. However, the results of phase II trials are inconclusive, failing to show a clear benefit from the addition of bevacizumab in terms of pCR as well as patients' outcomes (Willett et al. 2009; Crane et al. 2010). Although the multimodality approach (concurrent CRT followed by surgery) has improved the local control, the risk of metastatic recurrence remains high (30% rate), leading to death related to rectal cancer. Positive aspects of delivering CT in the neoadjuvant setting include less toxicity, higher rate of organ preservation, and increased downstaging. Several clinical studies regarding two new neoadjuvant paradigms have been recently published: NA-CRT followed by neoadjuvant chemotherapy (NAC) and NAC followed by NA-CRT. The phase II trial of Garcia-Aguilar et al. was the first study exploring the administration of NAC between CRT and surgery. Patients were randomized to four different arms defined by the number of NAC [zero, two, four, or six cycles of modified 5-fluorouracil and oxaliplatin (FOLFOX)]. The study was powered to detect a difference in pCR (primary endpoint); pCR was significantly higher in patients treated with six cycles of NAC compared to those who did not receive NAC (38 vs. 18%) (Garcia-Aguilar et al. 2015). Long-term disease-related outcomes have not been investigated in this latter study, but they are object of investigation in other similar trials with positive results. For instance, the Polish II trial (short-course RT + 3 cycle of FOLFOX vs. long-course RT with concurrent FOLFOX) showed significant improvement of 3-year OS (73 vs. 65%, $p = 0.046$) in the NAC arm (Bujko et al. 2016).

The second neoadjuvant paradigm consists of induction NAC followed by NA-CRT and then surgery; however, this strategy showed less encouraging results.

In the GCR3 phase II trial, patients were randomized to four cycles of capecitabine and oxaliplatin (CAPOX) before or after NA-CRT and no differences were observed in the two arms in terms of pCR (13 vs. 14%), DFS, and OS (Fernandez-Martos et al. 2015). Similar results were observed in EXPERT (Chua et al. 2010) and EXPERT-C trials (Dewdney et al. 2012) in which the NAC was represented by CAPOX and CAPOX plus cetuximab, respectively. On the other hand, in the CONTRE study, a single-arm trial in which patients received six cycles of FOLFOX followed by NA-CRT; all patients enrolled achieved R0 resection with a pCR rate of 33% (Perez et al. 2017).

Future directions of research in this field will likely focus on deescalating and escalating strategies, stratifying patients according to the risk factors with a possible impact on local and distant recurrence, such as nodal involvement, localization in the upper versus lower rectum, and response to the neoadjuvant treatment (i.e., bioselection of patients).

In case of intraperitoneal rectal cancer without nodal involvement, no benefit was observed of preoperative RT for local control and patients can be candidate to surgery. Patients obtaining a complete clinical response (cCR) evaluated by magnetic resonance imaging (MRI) assessment after NAC may not be good candidates for CRT as well. The ongoing phase II–III PROSPECT trial was designed to validate this approach (NCT01515787). Furthermore, patients with a cCR after NAC-CRT could be followed up omitting surgery. To better describe and classify the patterns of tumor response to treatments and understand the prognostic and predictive information, a standard tumor regression grading (TRG) system has been proposed. The Dworak TRG system describes five patterns of response: TRG4 (pCR or complete regression), TRG3 (near complete response, with very few tumor cells), TRG2 characterized by dominantly fibrotic changes, and TRG1/0 with dominant tumor mass with obvious/no regression (Dworak et al. 1997). The TRG investigated in a German rectal cancer trial strongly supports this idea: TRG 4 was associated with 5-year DFS of 86% versus 63% in patients with TRG0 (Rodel et al. 2005).

Additionally, several trials investigated the addition of new target therapies to NAC in order to improve its efficacy. A phase II clinical trial platform, known as NRG-GI002, has been designed to assess novel sensitizers to NAC and/or CRT in LARC (George et al. 2017). The first assessed drug was veliparib, a poly-ADP-ribose polymerase (PARP) inhibitor enhancing the effectiveness of RT by interfering with DNA repair mechanisms and thus killing or reducing tumor cells. The addition of veliparib did not reduce the amount of cancer present at the time of surgery. However, the combination treatment was safe and >90% of patients completed CT (Czito et al. 2017). The efficacy of addition of pembrolizumab to veliparib and neoadjuvant RT is still under evaluation (NCT02921256).The research is focusing also on the role of new biomarkers to predict the response to neoadjuvant treatment. TILs have a crucial role in tumor progression and survival outcome, and an antitumoral immune effect has been recognized to contribute to response to CT and RT. Multiple studies demonstrated the possible predictive value of TILs during NA-CRT. Matsutani et al. assessed the TIL density on pre- and posttreatment samples, showing that low-density TILs on both samples were associated with

poor response; of note, the authors observed an increased TIL density on posttreatment specimen (Matsutani et al. 2018). Additionally, oxaliplatin-based neoadjuvant therapy seems to induce a systemic immune response reducing the risk of recurrence, according to Kalanxhi et al. (2018), enhancing the immunogenic death of cancer cells. This study evaluated the serum levels of fms-like tyrosine kinase 3 ligand (Flt3), a factor involved in myelosuppression and antigen-presenting cell activation, showing that high Flt3 level reported after NA-CRT was linked to lower risk of recurrence. These results provided the rationale for the development of clinical trials investigating the role of immunotherapy in the neoadjuvant setting (avelumab single agent or in combination with CT (NCT03299660, NCT03854799); nivolumab single agent or in combination with CT after NA-CRT (NCT02948348, NCT03921684)). Over the last few decades, specific nomograms and scores have been developed in order to personalize this approach based on the risk of local and distant recurrence. The multivariate *Valentini nomogram*, which incorporates stage, type of surgery, pathological status of tumor (T) and nodes (N), gender and age, and type of treatment risk, represents the first nomogram used in this field. Finally, neoadjuvant rectal score (NAR), including nodal involvement (pN) and T downstaging as weighed and standardized variables, has been recently approved by the US National Cancer Institute as a surrogate endpoint of impact of NA-CRT in clinical trials (George et al. 2015).

## 1.3 Systemic Treatments for Advanced Disease in the Era of Personalized Medicine

The recognition of intrinsic subtypes and the rising identification of biomarkers of prognosis and prediction of response to treatments have opened the doors of personalized medicine for CRC. The use of monoclonal antibodies combined with standard CT is the standard approach in the treatment of advanced CRC. Efforts in the study of responders to targeted therapies have been pursued, but only a few predictive factors useful in the clinical practice have been discovered. For antiangiogenic agents, no biomarkers are available for clinicians in refining the patient's selection and reduce toxicity. Despite the initial response of patients to targeted therapies, such as cetuximab and panitumumab, in molecularly selected patients per *RAS* mutational status, survival gain and disease control improvement are still modest (Siravegna et al. 2015). The advent of immunotherapy along with the definition of hypermutating subtypes of CRC provided the rationale for delivering immunomodulating strategies of treatment such as immune-checkpoint inhibitors. The presence of different targetable alterations might improve the portfolio of treatment options when resistance occurs, but also emphasizes the need for treatment sequences.

### 1.3.1 The Evolving Role of Immune-Oncology of Colorectal Cancer

One of the most relevant predictive alterations of response for CRC is the mutational and functional status of the proteins of the DNA MMR. CRC harboring a deficit of the MMR (MSI-H) presents a distinct clinicopathological and molecular profile (Campbell et al. 2017). It is suggested that the high mutagenic potential of MSI-H tumors is able to enhance the generation of quality and effective tumor-associated neoantigens, capable of being recognized as "non-self" or "altered-self" from the immune system. MSI-H phenotype is described in two main settings: hereditary CRC syndromes, mainly due to germline mutations in the MMR genes, and sporadic alterations—primarily caused by promoter hypermethylation of the MMR protein MLH1. However, the action of immune system is based on multiple players and the tumor characteristics are often insufficient to predict entirely the outcome of patients (Mlecnik et al. 2016). In fact, an attempt to elucidate the relationship between MSI and TILs resulted in the development of an immunoscore, based on the quantification of cytotoxic and memory T cells in the core of the tumor and in the tumor's invasive margin. The immunoscore has been demonstrated to predict better the outcome than the MMR status alone, providing information both on the likelihood of the cancer to induce an immune activation and the functional antitumoral activation of the immune system (Llosa et al. 2015). Furthermore, patients with tumors exhibiting an MSI-H phenotype seem to derive less benefit in metastatic setting from CT, suggesting that the status of MMR may orient the development of strategies of treatment for a unique subgroup of CRC (Shulman et al. 2018).

The presence of a hypermutator phenotype is associated with an increased likelihood of effective immune response, regardless of the MMR. In fact, beyond the more common occurrence of MSI-H in CRC, other mutations can provide an immune-enhancing tumoral phenotype. Some non-MSI-H tumors exert an ultra-hypermutator phenotype when presenting defective replication repair of DNA, caused by mutations in the proofreading domain of the DNA polymerase ε (POLE) (Campbell et al. 2017). In clinical series, the POLE-mutated CRC accounted for 1% of all: patients with POLE category tumors were significantly younger than those with non-hypermutators and non-POLE-hypermutators (Hino et al. 2019). Currently, the American Food and Drug administration has approved three immune-checkpoint inhibitors for the treatment of patients with advanced and metastatic CRC previously treated with standard CT: the anti-PD1 pembrolizumab and nivolumab and the anti-CTLA4 ipilimumab, the latter combined with nivolumab (Morse et al. 2019). The approval was supported by the preliminary results of clinical trials, showing an interesting response pattern of tumors resistant or progressing to chemotherapies in around one half of the population treated (Le et al. 2015). In contrast, no responses were observed in stable MMR tumors. The use of combinations of immune-checkpoint inhibitors is expected to enhance the immune response and overcome emerging resistant mechanism to improve disease control. However, direct comparisons of single versus multiple agents are still awaited. Overall, primary resistance to immune-modulating agents remains common

in patients with MSI-H cancers, suggesting the need to improve patients' selection for treatment.

### 1.3.2 BRAF: Prognostication and Targetability

The mutational status of *BRAF* in CRC seems to dictate the prognosis of the tumors, recognizing a distinct entity with an aggressive biological behavior (Rajagopalan et al. 2002). *BRAF* mutations occur in less than 15% of all CRC. Despite a more common occurrence of the *V600E* mutation of *BRAF* in MSI-H tumors, a prognostic adverse significance has been described only for *BRAF*-mutated MMR unaltered tumors (microsatellite stable, MSS), accounting for 10% of all MSS tumors of the colon and the rectum (Clarke and Kopetz 2015). The presence of *BRAF* alterations confers a poorer outcome for resected patients in the curative setting, as evidenced in retrospective series (Zhu et al. 2016). A meta-analysis of seven phase III randomized clinical trials (1035 *BRAF*-mutated stage II and III CRC) showed a poorer OS (HR: 1.42, 95% CI: 1.25–1.60; $p < 0.00001$) and DFS (HR: 1.26, 95% CI: 1.07–1.48, $p = 0.006$) compared with $BRAF^{wt}$ patients. In this tumor, the mutation of *BRAF* is mutually exclusive to *KRAS* mutations—both representing critical alterations of the MAP kinase pathways for the multistep gastrointestinal carcinogenesis. Moreover, the prognostic role of *BRAF* mutational status is described in metastatic setting as well, suggesting a biological entity with reproducible behaviors in different settings (Lochhead et al. 2013).

The biological interplay and the analogy between *KRAS* and *BRAF* mutations as negative predictive biomarker have been extensively evaluated. Data from meta-analyses failed to clearly demonstrate a predictive role of *BRAF* when anti-EGFR are used, along with an inconclusive role in clinical decision-making, based on insufficient evidence (Rowland et al. 2015). Notwithstanding the scarce clinical evidence confirming a role of *BRAF* in determining resistance to anti-EGFR agents, an additive effect of dual EGFR and *BRAF* targeting has been described. In a recent interim analysis of the BEACON trial, the combination of binimetinib (selective inhibitor of MEK), encorafenib (*BRAF V600E* blocker), and cetuximab (anti-EGFR) showed a gain in median OS of +3.6 months (HR: 0.52, 95% CI: 0.39–0.70) and a response rate of +24% (Kopetz et al. 2019). Whether the single targeting of *BRAF* with cetuximab is enough or requires the MEK blockade is still the object of investigation, as encorafenib plus cetuximab provided a comparable OS gain in this trial and no formal comparison between the doublet and the triplet was performed. The role of *BRAF* in MSI-H tumors seems to be different. In fact, the occurrence of *BRAF* mutations seems to be contextual to the hypermutator phenotype, without a clear role in driving the tumorigenesis and conditioning the prognosis. In general, the MSI-H *BRAF*-mutated CRC responds to immunotherapy, with no difference with the $BRAF^{wt}$ MSI-H patients (Smeby et al. 2018).

### 1.3.3 Tailoring the Mechanisms of Resistance to Improve Patients' Selection

The exploration of novel mechanisms of resistance to therapies in CRC has resulted in the identification of possible new pharmacological targets. One emerging target in this setting is the oncoprotein HER2, widely described and studied in breast and gastric malignancies and responsible for the resistance to EGFR blockers in 5% of *KRAS*$^{wt}$ CRC (Richman et al. 2016). The analysis of this pathway and the known effective targetability in breast neoplasms provided the rationale for testing the anti-human epidermal growth factor receptor 2 gene (HER2) agents in preclinical models and in clinical trials. The phase-2 trial HERACLES assessed the combination of lapatinib plus trastuzumab in a cohort of 27 patients with HER2 overexpressed *KRAS*$^{wt}$ tumors, showing significant tumor shrinkage in one-third of the population. A similar result was achieved with trastuzumab plus pertuzumab in the MyPathway phase II basket study (Sartore-Bianchi et al. 2016). An alternative mechanism to target HER2 regardless of the ERBB2 oncogene addiction of the CRC has been suggested with the use of conjugated anti-HER2 antibodies; one study with trastuzumab deruxtecan showed an overall response rate (ORR) of 25% in this setting, after trastuzumab failure (Yoshino et al. 2018). However, the story of HER2 in CRC is still partially unexplored and needs more evidence to better understand the effective benefit of incorporating the HER2 blockade in the strategy of treatment, for example in earlier lines of therapy.

Attractive genomic alterations for targeting in CRC are gene translocations, described in less than 2% of this population. The most common gene fusions in CRC are described for *RET*, *NTRK*, *ALK*, or *ROS1*, all amenable to pharmacological targeting (Stransky et al. 2014). Anecdotal responses have been reported across different basket trials, using compounds capable of targeting the fusion products from *RET*, *NTRK*, *ALK*, and *ROS1* with different partners: larotrectinib, entrectinib, and ceritinib (Cocco et al. 2018). All these rare alterations have been correlated to a poorer benefit of anti-EGFR therapies and a worse outcome. Interestingly, no resistance to immunotherapy agents has been demonstrated when MSI-H tumors present concurrent gene translocations amenable to targeted therapies, despite not reproducible in all tumor types.

## 1.4 Current Clinical Management of Colorectal Cancer

### 1.4.1 Treatment of Colorectal Cancer: The Curative Setting

**Neoadjuvant Treatment**

Neoadjuvant treatment represents the gold standard in the management of locally advanced rectal cancer defined as T3 with clear circumferential resection margin (more than 1 mm from mesorectal fascia and *levator ani* muscle) evaluated by MRI, T1-T2 with N1 or N2. The NCCN guidelines 2019 recommend CRT with capecitabine or infusional 5-FU administered concomitantly with long-course

RT. The bolus of 5-FU and leucovorin is an option for patients who do not tolerate 5-FU in infusion and/or capecitabine. Short-course RT alone or followed by 12–16 weeks of oxaliplatin-based CT regimen (FOLFOX or CAPOX) or 5-FU/leucovorin could be valid options in selected cases. The use of short-term RT should be discussed in a multidisciplinary setting with a careful evaluation of the long-term toxicities. NAC with FOLFOX or CAPOX or 5-FU/leucovorin followed by standard concomitant CRT (fluoropyrimidines concomitantly to long-term RT) or short-term RT represents the possible choices (National Comprehensive Cancer Network 2019). The next step after CRT therapy provides radiological re-staging to plan surgery, when possible. In patients considered not operable, systemic therapy is the only choice. In cases of cCR—defined as no evidence of residual disease on digital rectal examination, endoscopic evaluation, and rectal MRI—a "watchful waiting" strategy could be adopted. Considering that risk of local or distant failure, omitting surgery has not yet well been characterized; decision-making should involve a multidisciplinary dedicated team and a clear discussion with the patient.

**Adjuvant Treatment**

- *Colon and rectal (intraperitoneal) cancer*
  The adjuvant treatment does not differ in case of colon or intraperitoneal rectal cancer. Adjuvant therapy should not be proposed after surgery in unselected patients, but only in case of nodal involvement (stage III) or high-risk patients without nodal involvement (stage II). The high risk is defined by the presence of at least one of poor prognostic factors, such as T4 tumor, bowel obstruction or perforation at diagnosis, poorly differentiated tumor (G3), lymphovascular or perineural invasion, elevated carcinoembryonic antigen (CEA) at diagnosis, low number of lymph nodes surgically removed (<12 nodes), and positive margins after surgery. In patients with high-risk stage II colon cancer, adjuvant CT with fluoropyrimidines (oral capecitabine and injective 5-FU plus leucovorin) showed to improve the DFS if compared to observation (Andre et al. 2004, 2009; Quasar Collaborative Group et al. 2007). However, it is important to define the microsatellite status of tumor in patients with stage II CRC and candidates to adjuvant CT before starting treatment. In fact, several trials reported a better prognosis in stage II colon cancer with MSI-H and dMMR, but low efficacy of adjuvant CT based on fluoropyrimidine agents (Sargent et al. 2010). The benefit of adding oxaliplatin in this setting has been under debate over the last few decades, but the evidence in literature on long follow-up period showed that schedules with oxaliplatin (FOLFOX or XELOX) do not improve DFS and OS in these patients when compared to fluoropyrimidine alone (Andre et al. 2009). Therefore, adjuvant CT could be considered in all patients with MSS stage II high-risk colon cancer after surgery, according to patient choice, age, and comorbidities.

  For patients with stage III cancer, the standard of care is represented by doublet CT with fluoropyrimidines and oxaliplatin (FOLFOX or XELOX), according to the results of three landmark trials (Andre et al. 2004, 2009; Kuebler et al. 2007; Haller et al. 2011). Indeed, each trial showed significant reduction in the risk of recurrence (23%) and improvement in DFS and OS with the adjuvant treatment.

Adjuvant CT should always be administered as soon as the patient is able to receive it, with better results if CT starts within 8 weeks after surgery. Regarding the optimal duration of adjuvant treatment, until few years ago the standard of care was represented by 6 months of chemotherapy. Recently, the IDEA collaboration investigated the possibility to use 3 months of FOLFOX/XELOX instead of six in order to reduce the incidence of peripheral neuropathy, which represents the main limitation of using oxaliplatin (Grothey et al. 2018). The results from the six randomized trials included in the IDEA collaboration work showed that neurotoxicity was lower in the 3 months arms, even if the primary endpoint of the trial (non-inferiority of 3 vs. 6 months) was not met. However, non-inferiority was observed in a selected group of patients with low-risk profile (pT1–3, N1) by using XELOX, whereas the endpoint was not met with FOLFOX. Based on these data, adjuvant treatment options for patients with low-risk stage III are XELOX for 3 months or FOLFOX for 3–6 months, according to the tolerability. For patients with stage III high risk (pT4, N1-2; any T, N2), the standard of care remains FOLFOX/XELOX for 6 months. An adjuvant treatment based on fluoropyrimidine single agent could be considered in patients with stage III CRC when oxaliplatin cannot be administered or is contraindicated. In those cases, capecitabine was shown to be equivalent to bolus of fluorouracil (Twelves et al. 2012). Adjuvant CT is recommended also for elderly patients (>65 years old) with similar results. However, schedules with single agents are preferred in this population, because the benefit of adding oxaliplatin in patients aged 70 years old or more is not clear and the risk of toxicity is higher (Haller et al. 2015). Other adjuvant schedules, such as the combinations with irinotecan as well as the addiction of other biological agents, are not recommended (Saltz et al. 2007; Van Cutsem et al. 2009). In fact, all the trials that evaluated irinotecan in the adjuvant setting failed to prove any benefit. Among biological agents, the use of bevacizumab in the adjuvant treatment in addition to FOLFOX or capecitabine did not show any improvement in treatment outcomes. Therefore, bevacizumab has no role in the adjuvant setting today (Allegra et al. 2011, 2013; De Gramont et al. 2012; Kerr et al. 2016). Similarly, cetuximab showed an increase in toxicities without any benefit in this setting regardless of *RAS* mutation and, therefore, is not indicated in the adjuvant treatment (Taieb et al. 2014, 2017).

- *Rectal cancer (extra peritoneal)*
  Adjuvant CT is recommended in each patient with stage II or III rectal cancer who did not receive neoadjuvant treatment, in those who received neoadjuvant radiotherapy alone, or in patients with poor histopathological features after surgery, such as positive margins, incomplete mesorectal resection, or perforation during surgery. The choice of treatments should be personalized and based on the pathological stage and type of neoadjuvant treatment administered. In case of stage II tumor after neoadjuvant approach and surgery, a single-agent treatment with fluoropyrimidines may be considered, whereas the addition of oxaliplatin is recommended in case of stage III. The duration of treatment depends on previous neoadjuvant therapy, with the possibility of shorter adjuvant CT (4 months) in case of preoperative therapy (6 months of treatment). In patients who did not

receive any neoadjuvant treatment, adjuvant treatment with concomitant CRT followed by CT could be an option for stage II (pT3 N0) tumors as well as the observation in case of good histopathological features. For stage III tumors, a combined treatment according to "sandwich strategy" (CT followed by concomitant CRT followed by CT for 6 months) is the standard of care (Smalley et al. 2006; O'Connell et al. 1994).

### 1.4.2 Management of Advanced Colorectal Cancer: The State of Art

The therapeutic goals in the management of advanced CRC are multiple and depend on the pattern of metastatic spread of disease and patient's characteristics. For each selected patient, a multimodal approach can be pursued with a curative intention; this subgroup includes patients with liver predominant and resectable metastatic disease or oligometastatic pattern of visceral spread. However, the primary goal of the treatment in case of metastatic disease is to optimize the quality of life and prolong survival, ensuring the best support in symptomatic control.

**Systemic Treatment**

Chemotherapy represents the first choice in case of advanced CRC. According to international guidelines, the treatment should be started as soon as possible after the diagnosis of metastatic disease, because clinical deterioration related to progression of disease may narrow the treatment choices due to lower tolerability of multiple systemic agents. A doublet based on fluoropyrimidines (oral capecitabine and injective 5-FU plus leucovorin), irinotecan, and oxaliplatin represents the standard of care in this field. Despite that the trials showed no efficacy differences by using oxaliplatin- (FOLFOX and XELOX) or irinotecan- (FOLFIRI) based doublets, the choice of the best treatment is mainly related to the different drugs' safety profile (Neugut et al. 2019). For instance, the use of oxaliplatin is associated with an increased risk of peripheral sensory neuropathy, whereas the irinotecan-based regimens are related to a higher incidence of diarrhea, dehydration, and neutropenia. The use of irinotecan has also been related to potentially severe adverse events, especially in case of alterations in the liver metabolism enzymes. In fact, patients who show polymorphisms of the enzyme UGT1A1—involved in the solubilization of xenobiotics and bilirubin via glucuronidation—showed severe toxicity after irinotecan, as a result of insufficient elimination of the drug and its higher systemic bioavailability (Takano and Sugiyama 2017).

Finally, biological agents, such as anti-EGFR (cetuximab and panitumumab) and anti-VEGF monoclonal antibodies, could be added to chemotherapy, according to molecular characteristics of the tumor.

- *Bevacizumab in the first-line setting*
  Bevacizumab is the first antiangiogenic agent approved for the management of metastatic CRC. The principal mechanism of action of bevacizumab is to neutralize the action of the VEGF, resulting in an antiangiogenic effect. The addition of

bevacizumab to CT has demonstrated to provide an adjunctive gain of +3.3 and +3.2 months in the overall and progression-free survival, respectively, in one pooled analysis of clinical trials (Kabbinavar et al. 2005). To date, the benefit of bevacizumab combined with triplets like FOLFOXIRI has not been assessed, as no comparison with or without the biological agent is available, as discussed below (TRIBE study). Furthermore, data from registry-based investigations showed an adjunctive benefit of bevacizumab when combined to irinotecan, failing to show any significant contribution in the oxaliplatin-containing doublets in the first-line setting (Macedo et al. 2012). Of note, the use of bevacizumab is associated with an increase in grades 3–4 hypertension, bleeding, thromboembolic events, and proteinuria, leading to increased treatment interruptions (Macedo et al. 2012). Such events include gastrointestinal perforation, pulmonary embolism, severe hypertension, gastrointestinal and cerebral hemorrhage, or vascular accident in up to 5% of the exposed population, leading to organ damage, including neurological permanent impairments (Taugourdeau-Raymond et al. 2012). The safety and clinical benefit of biologics, including bevacizumab in the elderly population, has been less addressed, generally underrepresented in clinical trials. The AVEX trial compared bevacizumab in combination with capecitabine or capecitabine alone in previously untreated patients 70 years or older, to assess the benefit and safety in elderly patients with metastatic CRC (Cunningham et al. 2013). The incorporation of bevacizumab improved PFS (median PFS 9.1 vs. 5.1 months), along with more adverse events related to bevacizumab (8% of venous thromboembolic events with bevacizumab vs. 4% and hemorrhage 25% vs. 7%). Overall, the combination can be proposed in this setting, using special prophylactic measures for patients at higher risk of cardiovascular accidents and prompting diagnostic and therapeutic interventions when such events are suspected. No predictive biomarkers have been identified to select the patients more likely to derive a benefit from the antiangiogenic therapy in addition to CT. However, some clinical and molecular features have been identified in the exposed patients and are associated with a larger benefit in terms of disease control. Firstly, patients experiencing hypertension (on-target side effect) seem to derive a greater benefit from bevacizumab. However, a gain in OS has not been uniformly confirmed (Dionisio de Sousa et al. 2016). Then, patients with an angiogenic switch in plasma protein profile have higher benefit in terms of PFS. The angiogenic switch can be determined by monitoring the plasma levels of angiogenic related cytokines such as hepatocyte growth factor (HGF), placental growth factor (PGF), macrophage chemoattractant protein-3 (MCP-3), MM-9, eotaxin, basic fibroblast growth factor (FGF), and interleukin 18 (IL-18) (Cubillo et al. 2019). Eventually, prognostic and predictive signatures have been proposed to understand the biological behavior of colorectal malignancies and enhance the targetability. A radiomic signature has been recently suggested, with capability to predict survival after 2 months from the start of bevacizumab through an imaging computer analysis (Dohan et al. 2019). Anyway, this radiomic signature warrants further prospective validations for clinical use. The role of bevacizumab in the perioperative treatment of metastatic resectable

disease is more controversial and debated. In fact, no gain in OS has been clearly showed (Gruenberger et al. 2015). Accordingly, the use of antiangiogenic agents is not recommended outside the metastatic disease.

- *Anti-EGFR therapy*
  The two approved anti-EGFR monoclonal antibodies for the management of metastatic CRC are cetuximab and panitumumab. Cetuximab is a chimeric murine-human monoclonal antibody and panitumumab is a fully humanized molecule. Both monoclonal antibodies have been studied in combination with F OLFOX or FOLFIRI for the initial treatment of $RAS^{wt}$ metastatic CRC. More robust data are available for the negative selection of patients deriving benefit from anti-EGFR, namely the ones presenting mutations in the codon 12 and 13 (exon 2) of the gene *KRAS*. However, the intrinsic resistance to anti-EGFR seems to be more complex spacing beyond exon 2 of *KRAS*. Indeed, retrospective evidence suggests an insensitivity of colorectal malignancies to anti-EGFR when presenting different types of mutations in *KRAS* (other than exon 2), *NRAS*, or *BRAF V600E*. Interestingly, these mutations are mutually exclusive in resistant tumors, suggesting an independent and non-overlapping role in the determination of a similar phenotype. Furthermore, new biomarkers have been reported, including mutations in the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α gene (*PIK3CA*) and amplification of *HER2* (see Chaps. 2 and 4 for more details). Additionally, exploratory evidence has questioned about the sidedness, hypothesizing that the primary localization of CRC, left or right colon side, could be related to a nonrandom distribution of specific mutagenic events, associated with a higher likelihood of resistance of right-sided tumors to anti-EGFR (Snyder et al. 2018). In fact, right-sided colorectal tumors seem to harbor more commonly alterations in *BRAF* and MAPK pathways. These mutations may contribute to the intrinsic molecular resistance to EGFR blockers (Loree et al. 2018). Patients presenting with $KRAS^{wt}$ left-sided tumors can derive benefit by the incorporation of anti-EGFR agents. For *KRAS* and $NRAS^{wt}$ right-sided CRC, frontline antivascular agents may be preferred, despite no conclusive evidence is available (Arnold et al. 2017). The incorporation of anti-EGFR monoclonal antibodies to CT has demonstrated a significant gain in overall response rate (ORR), PFS, and OS in $RAS^{wt}$ patients, according to the results of the landmark clinical trials: CRYSTAL (cetuximab and FOLFIRI), PRIME (FOLFOX and panitumumab), and OPUS (FOLFOX and cetuximab). The randomized phase III clinical trial CRYSTAL (FOLFIRI with or without cetuximab, $n = 1198$ patients) showed a gain in median OS of +3.5 months and +1.5 months in PFS for $KRAS^{wt}$ CRC (Van Cutsem et al. 2010). The results were consistently confirmed in the OPUS trial, with a double of the ORR in RAS wt CRC (+29%) (Bokemeyer et al. 2015). The randomized phase III clinical trial PRIME (FOLFOX with or without panitumumab, $n = 1183$ patients) provided also similar outcomes, with +1.4 and +4.2 months prolongation in PFS and OS, respectively (Douillard et al. 2014). Currently, the two anti-EGFR targeted agents should be selected based on clinical parameters and their safety profiles, as no formal comparison in the first-line setting has been performed.

Two distinct clinical trials have investigated whether bevacizumab or anti-EGFR should have priority in the frontline treatment of $KRAS^{wt}$ CRC, the FIRE-3, and PEAK trials. FIRE-3 compared FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab as first-line treatment for KRAS (exon 2) codon 12/13 wt CRC patients (Heinemann et al. 2014). The trial did not demonstrate an advantage to incorporate the frontline anti-EGFR or the anti-VEGF monoclonal antibody in terms of PFS and ORR. However, OS was significantly longer for patients enrolled in the cetuximab arm, 28.7 months and 25 months, respectively. The PEAK trial (FOLFOX plus either panitumumab or bevacizumab) showed a significant gain in PFS in the panitumumab arm, around +2.7 months (Rivera et al. 2017). Taken together, the results suggest that the anti-EGFR can be preferred in this setting.

- *Intensified regimens of treatments: The triplets*
  A separate chapter is represented by the triplet based on 5-FU/leucovorin, oxaliplatin, and irinotecan (FOLFOXIRI) in combination with bevacizumab. The phase III TRIBE trial evaluated the safety and efficacy of FOLFOXIRI plus bevacizumab versus FOLFIRI plus bevacizumab as first-line treatment for metastatic patients (Loupakis et al. 2014). The triplet chemotherapy provided a longer disease control with 2.4 months improvement in PFS and 12% in ORR. The subgroup analysis on the rarer *BRAF*-mutated variant of CRC ($n = 28$ patients) suggested a significant greater benefit, supporting a new hypothesis of work for a molecularly defined cancer with a unique aggressive clinical behavior. As expected, the triplet regimen resulted in more adverse events, especially in terms of grade 3 or 4 neurotoxicity, stomatitis, diarrhea, and neutropenia. Accordingly, the use of the triplet plus bevacizumab can be considered when rapid ORR for symptom relief or conversion of resectability is pursued in the strategy of management, tailoring patients with good performance status capable of tolerating such an intensive regimen. Despite that no automatic rule of prescription of the intensified regimen for the *BRAF*-mutated subpopulations should be endorsed, the triplet may have a role when *BRAF V600E* patients present a substantial systemic burden of disease and related symptoms, taking in mind the current conflicting findings based on exploratory investigations (Cremolini et al. 2018).
- *Deescalation in colorectal cancer*
  Deescalation treatment is an attractive strategy to maintain the response to more intensive regimens, addressing tolerability and safety on the longer period. In one randomized trial, the benefit of a maintenance deescalated regimen consisting of capecitabine and bevacizumab compared with no treatment was assessed after an initial treatment with XELOX plus bevacizumab (Goey et al. 2017). Almost one-quarter of the patients in the maintenance arm developed a clinically significant hand-foot syndrome, although quality of life seemed not to be meaningfully affected. Overall, the trial showed a longer disease control but failed to demonstrate a gain in OS, meaning that observation alone is a valid option for patients receiving a doublet regimen frontline up to six cycles or to the maximal tolerance and/or best response. A similar conclusion was reached using bevacizumab or

bevacizumab plus deescalated CT as maintenance, providing a non-inferior benefit on the disease control (Hegewisch-Becker et al. 2015). More recently, a meta-analysis of randomized controlled trials evaluating different maintenance strategies (fluoropyrimidines, fluoropyrimidines, and bevacizumab or only bevacizumab) was performed. The analysis showed no benefit of continuing full cytotoxic chemotherapy until progression vs. observation in terms of PFS; maintenance therapy showed a PFS but not an OS benefit, confirming the previous findings of single clinical trials (Sonbol et al. 2019). In *RAS*$^{wt}$ CRCs, a phase II randomized study compared single-agent panitumumab to panitumumab in association with leucovorin and 5-fluorouracil after a 4-month induction treatment with panitumumab and FOLFOX-4. Maintenance therapy with single-agent panitumumab alone was inferior to combination in terms of 10-month PFS (49 vs. 59.9%) (Pietrantonio et al. 2019). Differently, the randomized phase II MACRO2 TTD study compared single-agent cetuximab to modified FOLFOX plus cetuximab as maintenance therapy after first-line modified FOLFOX plus cetuximab (Aranda et al. 2018). There were no statistically significant differences both in PFS and OS between arms and the objective response rate was also similar (Aranda et al. 2018).

- *The clinical unmet need beyond the first line*
  The outcome of stage IV patients failing the frontline therapy is generally poor, prompting the need for research in a wide area of unmet needs. The use of novel antiangiogenic agents in the following lines, including bevacizumab, aflibercept, ramucirumab, and regorafenib, has been tested. The use of these agents in pretreated patients has provided a gain of survival of 1.5 months, on average, at the cost of more treatment-related toxicity (Van Cutsem et al. 2012; Tabernero et al. 2015; Grothey et al. 2013). A similar magnitude of benefit has been observed in this setting with the oral fluoropyrimidine TAS 102 (trifluridine/tipiracil) (Patel et al. 2019). Furthermore, approvals of two anti-PD1 and one anti-CTLA4 agent for CRC have opened the doors of immunotherapy for susceptible subtypes of CRC patients. The precision medicine approach permitted in this case to deliver the optimal treatment to the patients most likely to respond, namely those carrying a hypermutated tumoral phenotype, deriving a possible long-lasting benefit. This subgroup of patients accounts for nearly 8% of the entire population. Pembrolizumab provided objective responses in 40% of the population of MSI-H CRC patients, whereas patients presenting a tumor with pMMRP derived no benefit from immunotherapy (Le et al. 2015). In the phase II clinical trial Checkmate 142, the anti-PD1 nivolumab exerted responses in 31% of the MSI-H patients; the combination of ipilimumab with nivolumab showed higher rates of response, around 55%, in another cohort of the same study, suggesting an additive activity (Overman et al. 2018). Confirmatory trials are still awaited to ponder the effective magnitude of benefit of these agents, variously combined with either CT or other biological agents supposed to overcome several mechanisms of resistance to immune-checkpoint blockade.

## 1.5 Conclusions and Perspectives

Biomarker-driven treatment decision for patients with CRC is currently limited, as few biomarkers have been validated in prospective clinical trials. In an area of large uncertainties and often insufficient evidence to inform the clinical indication of new agents, patient-centered clinical trials informed by the best science are warranted in order to enhance the discovery of biomarkers and other tools capable of matching patients to effective therapies. The clinical research for CRC is oriented to respond to pragmatic questions to address patients' unmet needs, including deescalation and de-intensification of therapies. For instance, the identification of neoplasms with more intrinsic indolent behavior could prevent adjunctive treatments and toxicities. In addition, good biomarkers of response could have a pivotal role when tumor shrinkage is needed for the downstaging of advanced tumors, pursuing for more conservative locoregional approaches. In the plethora of proposed biomarkers and the various drugs under evaluation, clarification in well-designed trials is warranted, ensuring access to the best treatments of patients. In this context, the emergence of next-generation sequencing, liquid biopsy, single-cell mapping, and gut microbiota are promising advances to deliver precision oncology in the future (for additional reading, see Box 1.1).

**Box 1.1 Recommended Reading**

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(continued)

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# Colorectal Cancer Genetics: An Overview of the Actionable Building Blocks

2

Khalid El Bairi, Csongor Lengyel, Antonio Marra, and Said Afqir

**Abstract**

Colorectal cancer (CRC) is a heterogeneous disease in nature which is challenging for therapeutic decision-making. Genetics of CRC represents a potential framework for implementing personalized medicine in the management of this aggressive disease in order to select the best treatment for the right patient. Emerging data from recent reports and sequencing projects showed many actionable genetic alterations and provide evidence for treatment selection and prediction of drug response. Importantly, mutational status in CRC is currently considered by several international therapeutic guidelines as a scaffold for patients' stratification to improve survival outcomes. In this chapter, molecular pathways associated with CRC genetics in sporadic and hereditary CRC are discussed.

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**Keywords**

Colorectal cancer · Genetics · Biomarkers

## 2.1 Introduction

According to the latest GLOBOCAN report, CRC is the fourth most frequent cancer in both sexes combined and is still a leading cause of high mortality worldwide (Bray et al. 2018). CRC is one of the most biologically and clinically heterogeneous cancers. The epithelium of the colon and rectum has a high proliferation rate and therefore a hotspot for malignant degeneration (Aran et al. 2016). Dozens of driver genomic events and passenger mutations were described during the transformation of normal colonic epithelium to invasive tumors and have started to emerge as potential biomarkers for this disease (Vakiani 2017; Zarkavelis et al. 2017; Rodrigues et al. 2016). The sequence of germline and somatic oncogenetic alterations is well described of the driver events during the process of CRC initiation and progression (Burn et al. 2013; Kuipers et al. 2015). CRC follows a histological multistep tumorigenic process driving adenomas to invasive adenocarcinomas (Fearon and Vogelstein 1990). Notably, several carcinogenic pathways were found to drive these oncogenic sequential steps including chromosomal instability (CIN), microsatellite instability (MSI) which occurs sporadically in most of cases, and epigenetic alterations such as CpG island methylator phenotype (CIMP) (Bae et al. 2013; Vaiopoulos et al. 2014; El Bairi et al. 2018; Ma et al. 2018). Initial events including *APC* mutations activating the WNT and β-catenin signaling pathway, mutations in the *TP53*, *KRAS*/*BRAF*$^{V600E}$, MSI genes, and other emerging genetic alterations such as *EpCAM*, *TGF-β*/*SMAD, PI3K, PTEN*, and *HER2* are the most studied until this time (The Cancer Genome Atlas Network 2012; Kuipers et al. 2015; Vakiani 2017). Differences in mutational status and mechanisms of disease are believed to underlie the hallmarks of clinically distinct sporadic and hereditary CRCs (Fig. 2.1) (Hahn et al. 2016a; Fearon 2011; The Cancer Genome Atlas Network 2012). In this chapter, we discuss the current understanding of CRC genetics and its cross-talk with disease occurrence and progression. Moreover, we extend our discussion to the potential of these genetic alterations as prognostic and predictive biomarkers to improve patients' outcomes.

## 2.2 Colorectal Cancer Genetics: An Overview

### 2.2.1 Sporadic Colorectal Cancer

Sporadic CRC arises without known significant family history or germline mutations, and it is the most seen in the clinic (Carethers and Jung 2015; Aran et al. 2016). A genomic profiling using recent sequencing technology of sporadic CRC specimens provides important data regarding its genetics. A wide range of

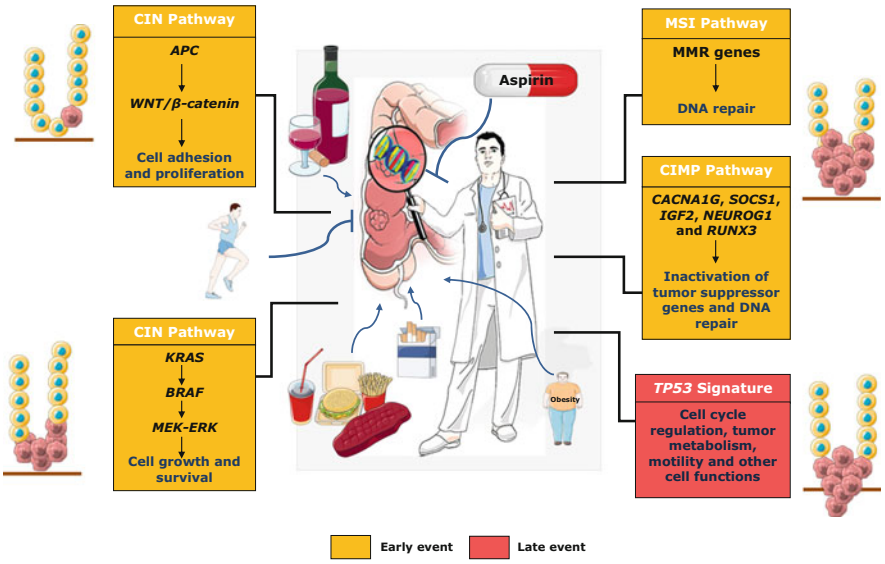


**Fig. 2.1** Overview of molecular pathways involved in sporadic and hereditary colorectal cancer. For comments, see text. *APC* adenomatous polyposis coli, *BRAF* v-raf murine sarcoma viral oncogene homolog B, *CACNA1G* calcium channel voltage-dependent T type alpha 1G subunit, *CIMP* CpG island methylator phenotype, *CIN* chromosomal instability, *DNA* deoxyribonucleic acid, *ERK* extracellular signal-regulated kinase, *IGF2* insulin-like growth factor 2, *KRAS* Kirsten rat sarcoma viral oncogene homolog, *MEK* MAPK/Erk kinase, *MSI* microsatellite instability, *NEUROG1* neurogenin 1, *RUNX3* runt-related transcription factor 3, *SOCS1* suppressor of cytokine signaling 1, *TP53* tumor phosphoprotein 53

somatic driver and passenger mutations and epigenetic changes were found in these tumors (reviewed elsewhere by El Bairi et al. 2018; Puccini et al. 2017; Rasool et al. 2014). It is believed that accumulation of mutations, chromosomal abnormalities, and epigenetic events confers a gain of function in oncogenes and loss of function in tumor suppressor genes which increase the proliferation rate and therefore a progress toward preinvasive tumors (Carethers and Jung 2015; Vogelstein et al. 2013). Typically, the most frequent and constant earliest event in CRC pathogenesis related to the CIN pathway is the occurrence of APC dysfunction (a key negative regulator of the WNT/β-catenin homeostasis) (Liang et al. 2013; Powell et al. 1992; Pino and Chung 2010; Al-Sohaily et al. 2012). The loss of heterozygosity in this tumor suppressor gene leads to the accumulation of β-catenin (encoded by *CTNNB1* gene, reviewed by Rosenbluh et al. 2014), a protein known for its role in cell adhesion and proliferation (Rosenbluh et al. 2014; Kim et al. 2013). In this direction, loss of both alleles of the gene is necessary to lose its function, a mechanism known as Knudson's two-hit model (Knudson and Strong 1972; Moolgavkar and Knudson 1981; Berger et al. 2011). Importantly, CIN was shown recently to drive and promote cancer metastasis to distant organs by chronically activating the immune pathways through a cytosolic DNA response (Bakhoum et al. 2018).

*KRAS* point mutations and $BRAF^{V600E}$ activation are other driver events and the most frequent in the adenoma-carcinoma process (Al-Sohaily et al. 2012). Deregulation of *KRAS* oncogene induces a pleiotropic constitutive activation of downstream signaling effectors such as RAF, MEK, and ERK and was found to be associated with a pivotal role in cell growth, survival, vesicle trafficking, invasion, and migration (Pino and Chung 2010). Interestingly, *KRAS* alteration is considered as a potent prognostic and predictive biomarker as demonstrated by many recent meta-analyses (Li et al. 2014a; Brudvik et al. 2015; Chen et al. 2013; Rowland et al. 2016; Sorich et al. 2015). The loss of *TP53,* a tumor suppressor gene frequently mutated in most of human cancers (Leroy et al. 2014), is another example of the observed alterations during colorectal carcinogenesis (Liu et al. 2015; Naccarati et al. 2012; Carethers and Jung 2015). Its alteration in CRC has been reported in various studies and is considered as a late event in tumor progression process (Al-Sohaily et al. 2012). *TP53* controls hundred of genes related to many important tumor signaling pathways such as tumor metabolism, cell cycle, tumor dormancy, angiogenesis, motility, and many other cell functions (Pino and Chung 2010) (details about this "guardian of the genome" can be found in the *TP53* databases: http://p53.fr and http://p53.iarc.fr). Moreover, new genes emerged recently as additional alterations in CRC. These include *COX*, *WNT*, *PIK3CA*, *TGFBR2*, *ARID1A*, *ERBB2*, and other low prevalent gene mutations (see reviews by Kuipers et al. 2015; Pino and Chung 2010).

In addition to the previously discussed CIN signaling pathway, MSI is another driving hallmark in CRC (Kawakami et al. 2015; Kloor et al. 2014; Yamamoto and Imai 2015). Of note, microsatellites are repetitive sequences of nucleotides that may experience errors during DNA replication (Al-Sohaily et al. 2012). Correction of these errors implicates the mismatch repair system called MMR that contains many genes encoding for DNA repair enzymes. Mutations in MMR genes explain and characterize the observed alternative and hypermutable pathway called MSI. Somatic mutations and gene silencing by hypermethylation were found in most of the MMR system genes (*MSH2*, *MSH6*, *MLH1*, *PMS1*, *PMS2*, *MLH3*, and *MSH3*) which account for about 12% of sporadic CRC (Sameer et al. 2014; Poulogiannis et al. 2010; Boland and Goel 2010). CRC with MSI phenotype tends toward poor differentiation, proximal location, high density of tumor-infiltrating lymphocytes (TILs), few distant metastases, and a good prognosis but poor chemoresponse to adjuvant 5-fluorouracil-based therapy (Boland and Goel 2010; Kloor et al. 2014). MSI in CRC is divided into two distinct subtypes: MSI-high with at least two positive markers (usually MLH1) and MSI-low with one positive marker; tumors without gene instability are called microsatellite stable (MSS) (Al-Sohaily et al. 2012). Typically, sporadic CRC presents an MSI-high associated with mutations in *BRAF* oncogene (negative in Lynch syndrome; see further), in addition to a concomitant hypermethylated phenotype known as CIMP (methylated *CACNA1G*, *SOCS1*, *IGF2*, *NEUROG1*, and *RUNX3*), as well as few mutations in *TP53* and *KRAS* (Al-Sohaily et al. 2012; El Bairi et al. 2018) (additional data about other mutations and cytogenetic changes in sporadic CRC are summarized in Table 2.1).

**Table 2.1** Summary of additional genetic and cytogenetic alterations in sporadic colorectal cancer

| Genetic alteration | Mechanism | Chromosomal Location[a] | Cell function and findings | References |
|---|---|---|---|---|
| *Genetic and epigenetic alterations* | | | | |
| *EGFR* | Polymorphisms | 7p11.2 | • Cell proliferation, survival, and angiogenesis | Martinelli et al. (2010), Poole et al. (2011) |
| *PIK3CA* | Point mutations | 3q26.32 | • Cell survival and growth | Zhu et al. (2014), Zhang et al. (2015), Samuels et al. (2004), Abubaker et al. (2008), Miyaki et al. (2007) |
| *VEGF* | Polymorphisms | 6p21.3 | • Angiogenesis and vascular permeability<br>• *VEGF* polymorphisms may play a role in the development of CRC | Maltese et al. (2009), Jannuzzi et al. (2015), Jang et al. (2013), Slattery et al. (2014)<br>Meta-analyses: Zhou et al. (2011), Zhao et al. (2012)<br>Review: Hansen and Jakobsen (2011) |
| *MCC* | Promoter hypermethylation<br>Point mutations (substitutions) | 5q21 | • Cell cycle arrest | Kohonen-Corish et al. (2007), Starr et al. (2009), Kinzler et al. (1991) |
| *CTNNB1* | Point mutations (substitutions) | 3p22.1 | • Regulation of WNT pathway<br>• Cell adhesion and migration<br>• *CTNNB1* alteration seems to be of minor importance in sporadic CRC<br>• *CTNNB1* mutations seem to occur more frequently in the proximal colon | Schneider et al. (2011), Lüchtenborg et al. (2005), Sygut et al. (2012) |
| *ARID1A* | Frameshift, nonsense, missense, splice site, and silent mutations | 1p36.11 | • Transcription regulation and chromatin remodeling<br>• *ARID1A* loss lacks prognostic value in stage I/II CRC | Cajuso et al. (2014), Mathur et al. (2017), Lee et al. (2016) |

(continued)

**Table 2.1** (continued)

| Genetic alteration | Mechanism | Chromosomal Location[a] | Cell function and findings | References |
|---|---|---|---|---|
| *MYC* | Amplification | 8q24.21 | • Cell cycle progression and differentiation<br>• MYC overexpression is correlated with metastatic phenotypes | Sánchez-Pernaute et al. (2005), Ozakyol et al. (2006) |
| *SOX9* | Copy number gain, frameshift and nonsense mutations | 17q24.3 | • Cell differentiation and cell stemness<br>• *SOX9* regulates cell plasticity and metastasis in CRC | The Cancer Genome Atlas Network (2012), Javier et al. (2016) |
| *IGF2* | Loss of imprinting[b] and copy number gain | 11p15.5 | • Cell growth, survival, and metabolism<br>• IGF2 loss of imprinting is a possible diagnostic, prognostic, and predictive biomarker for CRC | The Cancer Genome Atlas Network (2012), Zanella et al. (2015), Tian et al. (2012), Cheng et al. (2010), Ito et al. (2008), Baba et al. (2010) |
| *COX2* | Polymorphisms | 1q31.1 | • Inflammation<br>• *COX2* −765G>C polymorphism may be a risk factor of CRC in Asian patients | Peng et al. (2014) (a meta-analysis of case-control studies) |
| *MTHFR* | Polymorphisms | 1p36.22 | • Folates metabolism<br>• *MTHFR* 677 TT homozygous genotype significantly decreases the risk of CRC in Asians<br>• MTHFR polymorphisms might modify CRC risk in some ethnicities | Guo et al. (2014) (a meta-analysis); Haerian and Haerian (2015) |

(continued)

**Table 2.1** (continued)

| Genetic alteration | Mechanism | Chromosomal Location[a] | Cell function and findings | References |
|---|---|---|---|---|
| *CDH1* | Methylation and polymorphisms | 16q22.1 | • Epithelial-to-mesenchymal transition<br>• *CDH1* gene polymorphisms and methylation might affect the susceptibility of CRC | Smith et al. (2015), Govatati et al. (2014), Wang et al. (2012a), Geng et al. (2012), Li et al. (2014b) |
| *Cytogenetic alterations* | | | | |
| 8p loss | Deletion | 8p21 | • Deregulation of genes in this locus appears to be a hotspot for tumor progression and metastatic potential | Mourra et al. (2008), Macartney-Coxson et al. (2008) |
| 17p loss | Deletion | Not applicable (NA) | • Cell cycle arrest, tumor metabolism, cell death, etc.<br>• Loss of this locus which contains the *TP53* gene is a late event in the process of CRC<br>• This loss is an independent factor of poor outcomes | Risio et al. (2003), Watatani et al. (1996), Sánchez-Pernaute et al. (2005) |
| 18q loss | Deletion | NA | • Metastasis modulation and cell migration<br>• This region contains many tumor suppressor genes such as *DCC*, *SMAD2*, and *SMAD4*<br>• Loss of heterozygosity in this locus is a biomarker for poor prognosis of CRC | Wang et al. (2010), Pilozzi et al. (2011), Bertagnolli et al. (2011) |
| 13q gain | Amplification | NA | • 13q amplification appears to have candidate genes that may confer an aggressive CRC | Fensterer et al. (2007) |

(continued)

**Table 2.1** (continued)

| Genetic alteration | Mechanism | Chromosomal Location[a] | Cell function and findings | References |
|---|---|---|---|---|
| Gains in 20q, 13q, 7p, and 8q and losses in 18q, 8p, 1p, and 18p | Amplification and deletion | NA | • These genomic alterations may be a morphological signature for metastatic CRC to the liver | Korn et al. (1999) |
| Losses in 8p, 17p, 18p, or 18q and gains in 8q and 20q | Amplification and deletion | NA | • CGH-based analysis found that MSI-high tumors have DNA copy number alterations frequently involving 8q | Nakao et al. (2004) |

*ARID1A* AT-rich interactive domain 1A, *CDH1* cadherin 1, *CGH* comparative genomic hybridization, *COX2* cyclooxygenase 2, *CRC* colorectal cancer, *CTNNB1* catenin-β1, *DCC* deleted in colorectal cancer, *EGFR* epidermal growth factor receptor, *IGF2* insulin-like growth factor 2, *MCC* mutated in colorectal cancer, *MSI* microsatellite instability, *MTHFR* methylenetetrahydrofolate reductase, *MYC* v-myc avian myelocytomatosis viral oncogene homolog, *PIK3CA* phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha, *SMAD2* mothers against decapentaplegic homolog 2, *SMAD4* mothers against decapentaplegic homolog 4, *SOX9* SRY-box 9, *VEGF* vascular endothelial growth factor, *WNT* wingless-type MMTV integration site family member

Detailed tables about CRC genetic alterations can be found in Kuipers et al. (2015) and Migliore et al. (2011)

[a]Chromosomal location was retrieved using the HGNC database (https://www.genenames.org) and from the cited corresponding reference

[b]Mechanisms of loss of imprinting can be found in details in two recent reviews by Leick et al. (2012) and Uribe-Lewis et al. (2011)

### 2.2.2 Colorectal Cancer-Associated Hereditary Syndromes

Several Mendelian syndromic alterations have been described as predisposing factors for hereditary CRC (Table 2.2). Based on the presence of multiple polyps, these syndromes can be separated into non-polyposis and polyposis CRC (Ma et al. 2018). Notably, advances in sequencing techniques and genome-wide association studies (GWAS) have decoded novel variants associated with risk to develop CRC in addition to the already known syndromes.

#### 2.2.2.1 Lynch Syndrome (Hereditary Non-polyposis CRC (HNPCC))

Historically, Lynch syndrome was first described in 1966 by Lynch et al. (1966). Since then, a remarkable amount of the literature elucidated its molecular pathogenesis. Lynch syndrome (OMIM: 120435) is a high penetrant autosomal dominant non-polyposis hereditary disease and the most studied as a risk factor for hereditary CRC until now. This syndrome is caused by heterozygous germline mutations in the MMR tumor suppressor genes (*MLH1*, *MSH2*, *MSH6,* and *PMS2*) which drive

**Table 2.2** Hereditary syndromes and novel emerging genes predisposing to colorectal cancer

| Hereditary gene alteration | | Chromosomal location[a] | Inheritance[a] | Signaling pathway | Related syndrome | References |
|---|---|---|---|---|---|---|
| DNA mismatch repair genes | *MLH1* | 3p22.2 | Autosomal dominant (AD) | DNA repair | Lynch syndrome (also known as hereditary non-polyposis CRC (HNPCC)) (OMIM: 120435) | Seth et al. (2018), Carethers and Stoffel (2015) |
| | *MSH2* | 2p21-p16.3 | AD | DNA repair | | |
| | *MSH6* | 2p16.3 | AD | DNA repair | | |
| | *PMS2* | 7p22.1 | AD | DNA repair | | |
| *EpCAM* | | 2p21 | AD[b] | Cell adhesion and epithelial-to-mesenchymal transition (EMT) | | Ligtenberg et al. (2009), Ligtenberg et al. (2013), Kempers et al. (2011) |
| *BRCA1 and BRCA2* | | BRCA1: 17q21.31 BRCA2: 13q13.1 | [b] | DNA double-strand break repair | | Yurgelun et al. (2015a) |
| *TGFBR2* | | 3p24.1 | [b] | Regulation of cell growth | | Pinheiro et al. (2015) |
| *PTEN* | | 10q23.31 | AD | Gene repair and cell cycle regulation | Cowden syndrome (OMIM: 158350) | Pilarski et al. (2013), Jelsig et al. (2014), Gammon et al. (2016) |
| *APC* | | 5q21 | AD | Regulation of β-catenin/WNT pathway | Familial adenomatous polyposis (FAP) (OMIM: 175100) | Jasperson and Burt (2015), Ma et al. (2018) |
| | | | | | Attenuated familial adenomatous polyposis (AFAP) (OMIM: 175100) | Jahng et al. (2013), Talseth-Palmer (2017) |
| *SMAD4* | | 18q21.2 | AD | Cell proliferation and differentiation | Juvenile polyposis syndrome (OMIM: 174900) | Cichy et al. (2014) |
| *BMPR1A* | | 10q23.2 | | | | |

(continued)

**Table 2.2** (continued)

| Hereditary gene alteration | Chromosomal location[a] | Inheritance[a] | Signaling pathway | Related syndrome | References |
|---|---|---|---|---|---|
| *MUTYH* | 1p34.1 | Autosomal recessive (AR) | Repair of DNA damage caused by reactive oxygen species (ROS) | MUTYH-associated polyposis | Talseth-Palmer (2017) |
| *NTHL1* | 16p13.3 | AR[b] | DNA damage repair | NTHL1-associated polyposis (OMIM: 616415) | Talseth-Palmer (2017) |
| *STK11* | 19p13.3 | AD | Cell proliferation and polarity | Peutz–Jeghers syndrome (OMIM: 175200) | Beggs et al. (2010) |
| *AXIN2* | 17q24.1 | AD | Regulation of β-catenin/WNT pathway | Oligodontia-colorectal cancer syndrome (OMIM: 608615) | Lammi et al. (2004), Mazzoni and Fearon (2014) |
| *POLD1* | 19q13.33 | AD[b] | DNA replication and repair | Polymerase proofreading-associated polyposis | Talseth-Palmer (2017), Buchanan et al. (2017b), Rosner et al. (2018); for review, see: Bourdais et al. (2017) |
| *POLE* | 12q24.33 | | | | |
| *GREM1* | 15q13.3 | AD[b] | Regulation of TGF-β and sonic hedgehog signaling pathways | Hereditary mixed polyposis syndrome (OMIM: 601228) | Plesec et al. (2017), Lieberman et al. (2017), Jaeger et al. (2012) |
| *TP53* | 17p13.1 | AD | Cell cycle, tumor metabolism, tumor dormancy, angiogenesis, motility, and many other cell functions | Li-Fraumeni syndrome (OMIM: 151623) | Yurgelun et al. (2015b), Stoffel (2016), Amadou et al. (2018) |
| *BRCA2* | 13q13.1 | AD[b] | DNA double-strand break repair | Familial colorectal cancer type X | Garre et al. (2015; reviewed by: Nejadtaghi et al. 2017) |
| *FAN1* | 15q13.3 | AD[b] | DNA repair of interstrand cross-links | Hereditary non polyposis colorectal cancer | Seguí et al. (2015) |

| | | | | | |
|---|---|---|---|---|---|
| *BUB1* and *BUB3* | BUB1: 2q13<br>BUB3:<br>10q26.13 | [b] | Spindle assembly checkpoint control | Non polyposis early-onset colorectal cancer | de Voer et al. (2013), Hahn et al. (2016b; critically reviewed by: Broderick et al. 2017) |
| *RNF43* | 17q23.2 | AD[b] | Regulation of DNA damage response and WNT pathway | Serrated polyposis syndrome (OMIM: 617108) | Yan et al. (2017), Buchanan et al. (2017a), Taupin et al. (2015) |

*APC* adenomatous polyposis coli, *AXIN2* axin 2 (also known as conductin), *BMPR1A* bone morphogenetic protein receptor 1A, *BRCA1* breast cancer 1, *BRCA2* breast cancer 2, *BUB1* BUB1 mitotic checkpoint serine/threonine kinase (also known as budding uninhibited by benzimidazoles 1), *BUB3* mitotic checkpoint protein (also known as budding uninhibited by benzimidazoles 3), *CRC* colorectal cancer, *EpCAM* epithelial cell adhesion molecule, *FAN1* FANCD2/FANCI-associated nuclease 1, *GREM1* gremlin 1 (DAN family BMP antagonist), *MLH1* mutL homolog 1, *MSH2* mutS homolog 2, *MSH6* mutS homolog 6, *MUTYH* mutY DNA glycosylase, *NTHL1* nth like DNA glycosylase 1, *PMS2* postmeiotic segregation increased (*S. cerevisiae*) 2, *POLD1* DNA polymerase delta 1, *POLE* DNA polymerase epsilon, *PTEN* phosphatase and tensin homolog, *RNF43* ring finger protein 43, *SMAD4* mothers against decapentaplegic homolog 4; *STK11* serine/threonine kinase 11, *TGFBR2* transforming growth factor beta receptor 2, *TP53* tumor protein p53, *WNT* Wingless-type MMTV integration site family member

[a]Chromosomal location and inheritance were retrieved using the HGNC database (https://www.genenames.org), the Online Mendelian Inheritance in Man® database (http://omim.org), as well as the cited references

[b]The current knowledge of the mode of inheritance is still limited. Additional data about novel emerging genes can be found in a recent review by Valle (2017)

adenomatous polyps to carcinoma (Carethers and Stoffel 2015). CRC patients with these inherited mutations have lost the ability to repair the accumulation of single base pair mismatches, insertions, as well as deletions during DNA replication which leads to the MSI phenotype (Lynch et al. 2015). Interestingly, in a major recent advance, germline deletion of *EpCAM* (a key player during the epithelial-to-mesenchymal process), *TGFBR2*, *BRCA1*, and *BRCA2* mutations appear to increase the risk of this syndrome, but it still poorly understood (Kempers et al. 2011; Ligtenberg et al. 2009, 2013; Yurgelun et al. 2015a). From a histopathological point of view, analysis of cancer tissues from Lynch syndrome patients exhibits some characteristics such as the presence of poor differentiation and mucinous features, TILs, and Crohn's like reaction (Shia et al. 2013). Lynch syndrome is dichotomized into type I with colonic site-specific tumors and type II with extracolonic tumors (endometrium, ovary, biliary tract, stomach, skin (Muir–Torre syndrome: see OMIM: 158320), etc.) (Lynch et al. 2015). Lynch syndrome patients who do not fulfill the Amsterdam Criteria (presence of MMR germline mutations) are classified as "Lynch-like" and "familial colorectal cancer type X," characterized by the lack of disease-predisposing MMR alterations (Valle 2017; Rodriguez-Soler et al. 2013, reviewed by Dominguez-Valentin et al. 2015). Details about pathogenesis, current diagnostic guidelines, and management of Lynch syndrome are discussed in a recent open access review by Kastrinos and Stoffel (2014), in the OMIM database: 120435, by Umar et al. (2004) and the US Multi-Society Task Force on Colorectal Cancer as well (Giardiello et al. 2014).

#### 2.2.2.2 Familial Adenomatous Polyposis

Phenotypically, familial adenomatous polyposis (FAP) (OMIM: 175100) is characterized by the occurrence of hundreds to thousands of colonic polyps which inevitably progress into CRC and transmitted in an autosomal dominant manner (Aihara et al. 2014; Ma et al. 2018). At the histopathological level, these polyps display dysplastic crypts known as aberrant crypt foci and are considered as the "lighter" of carcinoma (Ma et al. 2018). FAP is a highly penetrant syndrome caused by germline variants in the *APC* gene (Jasperson and Burt 2015; Ma et al. 2018). In FAP, the most frequent mutations in the *APC* gene are nonsense and frameshift inactivating variants (Nieuwenhuis and Vasen 2007; Ma et al. 2018). Consequently, a truncated APC protein is considered as the initiating molecular event of the malignant transformation of the polyps. Furthermore, mutations in the 5′ and 3′ regions of this gene were associated with low number of synchronous adenomas (less than 100) (Nieuwenhuis and Vasen 2007; Su et al. 2000). This condition is defined as attenuated familial adenomatous polyposis (AFAP) (for practical guidelines, see Syngal et al. 2015).

#### 2.2.2.3 Hamartomatous Hereditary Syndromes

Hamartomatous hereditary syndromes (HHS) are inherited syndromes in an autosomal dominant pattern and include Cowden syndrome (OMIM: 158350), Peutz–Jeghers syndrome (OMIM: 175200), and juvenile polyposis syndrome (OMIM: 174900) (Jelsig et al. 2014). These syndromes are well known by the presence of

multiple hamartomatous polyps in the digestive tract and extraintestinal tumor locations as well (Jelsig et al. 2014). Patients with these syndromes have an increased risk of developing a large spectrum of tumors including CRC (Campos et al. 2015).

**Cowden Syndrome**
In the case of Cowden syndrome, inactivating small deletions and insertions and point mutations in *PTEN* tumor suppressor gene are the most seen in sequencing reports (Jelsig et al. 2014). This gene is known to be a key regulator of the PI3K/Akt pathway in the downstream of the EGFR signaling pathway (Molinari and Frattini 2013; Jelsig et al. 2014). Mutations in this gene are involved in the upregulation of cell growth and survival and therefore a sustained proliferative signaling for cancer initiation and progression (Hanahan and Weinberg 2011).

**Peutz–Jeghers Syndrome**
Peutz–Jeghers syndrome is inherited in an autosomal dominant manner, which can be distinguished from other hamartomatous syndromes by the presence of multiple mucocutaneous melanotic pigmentations and smooth muscle component in each polyp (Campos et al. 2015). Mutations in *STK11* gene encoding for a serine/threonine kinase are associated with cell cycle and polarity (reviewed systematically in detail by Beggs et al. 2010). Truncating mutations in this gene are suggested to increase the risk to develop malignancy (Beggs et al. 2010).

**Juvenile Polyposis Syndrome**
Juvenile polyposis syndrome is another risk to develop CRC (Cichy et al. 2014). Heterozygous germline mutations in *BMPR1A* and *SMAD4* were found to predispose to this disease (Cichy et al. 2014). Both genes are involved in TGF-β canonical pathway which is required for intestinal epithelium specialization. Recently, patients with mutated *SMAD4* were found to have more aggressive cancers than those with *BMPR1A* (Aytac et al. 2015). Other emerging Mendelian CRC-associated syndromes are summarized in Table 2.2.

The picture of somatic and hereditary CRC is far from complete. Remarkably, emerging NGS technology and new large sequencing projects such as the Cancer Genome Atlas Project (TCGA) (Weinstein et al. 2013; The Cancer Genome Atlas Network 2012) and the Human Cancer Pathology Atlas (Uhlen et al. 2017; El Bairi et al. 2017a) are a new milestone which identified more novel and unclassified variants and pathogenic mutations. These large-scale studies revealed many tumor signatures allowing new functional subclassifications of CRC.

### 2.2.3 Emerging Data from the Cancer Genome Atlas Project (TCGA)

The TCGA collaborative project (available at: https://cancergenome.nih.gov/) was launched in 2005 by the NIH (National Institutes of Health) to explore genomic alterations in human tumors (https://cancergenome.nih.gov/abouttcga/overview/

history). Since then, genomic big data of human tumor tissues (2.5 petabytes from more than 11,000 cancer patients) were analyzed and successfully characterized, catalogued, and made publically available for cancer researchers and oncologists (Tomczak et al. 2015). So far, TCGA network provides genomic profiles of 33 cancers until now including CRC. CRC-related TCGA project analyzed 276 tumor samples using large-scale exome sequencing, copy number variation, and transcriptomic and epigenetic expression techniques (The Cancer Genome Atlas Network 2012). Interestingly, examination of mutation rates in this cancer allowed a subclassification into hypermutated and non-hypermutated tumors (Fig. 2.2).

#### 2.2.3.1 Hypermutated Colorectal Cancer

These tumors had a mutation rate $>12/10^6$ bases and represented 16% of all sequenced CRCs (The Cancer Genome Atlas Network 2012). Three-fourths of these tumors were MSI-high, with silenced *MLH1* and hypermethylation patterns, and one-fourth had somatic alterations in MMR genes as well as mutations in DNA proofreading *POLE* gene (The Cancer Genome Atlas Network 2012). Hypermutated tumors included frequent mutations in *ACVR2A* gene (63%) which is a key gene in the TGF-β cell proliferation and differentiation pathway followed by *APC* (51%), *TGFBR2* (51%), $BRAF^{V6OOE}$ (46%), *MSH3* (40%), and *MSH6* (40%) (The Cancer Genome Atlas Network 2012). These tumors had fewer DNA copy number alterations (The Cancer Genome Atlas Network 2012; reviewed by: Müller et al. 2016). *TP53* and *APC* mutations were less frequently observed compared to non-hypermutated tumors (20% vs. 60%, $p < 0.0001$ and 51% vs. 81%, $p = 0.0023$, respectively) (The Cancer Genome Atlas Network 2012). This hypermutated profile showed better survival which may be a prognostic signature of this CRC subtype. In addition, deregulation of WNT signaling pathway was observed in 97% of hypermutated CRC with promising perspectives for pharmacological inhibition (The Cancer Genome Atlas Network 2012). In this perspective, several WNT pathway inhibitors are being investigated in several clinical trials (NCT02020291, NCT01351103, NCT02413853, and NCT02278133).

#### 2.2.3.2 Non-hypermutated Colorectal Cancer

In this non-hypermutated group (84%; low mutation rate $<8.24/10^6$ bases), *APC* (81%), *TP53* (60%), *KRAS* (43), *TTN* (31%), *PIK3CA* (18%), *FBXW7* (11%), *SMAD4* (10%), *TCF7L2* (9%), *NRAS* (9%), *TCF7L2* (9%), and *FAM123B* (7%) were the most frequently mutated genes (The Cancer Genome Atlas Network 2012). Expectedly, *KRAS* and *NRAS* had mutations in codons 12, 13, and 61. Moreover, a high number of DNA somatic copy number variants and novel role of mutated *SOX9* gene in human cancers were observed. Importantly, non-hypermutated tumors from colon and rectum had similar genomic profile (The Cancer Genome Atlas Network 2012).

In conclusion, according to the TCGA project, CRC genomic analysis shows that alterations in TP53, WNT, TGF-β, MAPK, and PI3K signaling pathways may indeed yield promising targets for cancer drug discovery (Fig. 2.2). However, the

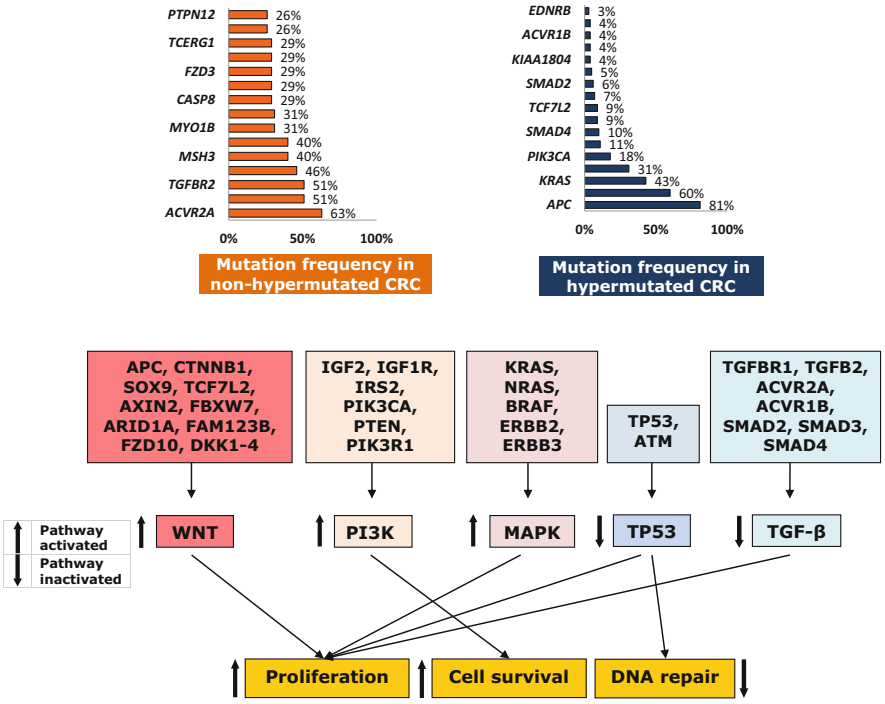


**Fig. 2.2** Mutation frequency (significantly mutated genes) and their related altered pathways in colorectal cancer according to the TCGA project after removal of non-expressed genes. Data from: The Cancer Genome Atlas Network (2012). *ACVR1B* activin A receptor type 1B, *ACVR2A* activin A receptor type 2A, *APC* adenomatous polyposis coli, *ARID1A* AT-rich interaction domain 1A, *ATM* serine/threonine kinase, *AXIN2* axin 2, *BRAF* v-raf murine sarcoma viral oncogene homolog B, *CASP8* caspase 8, *CDC27* cell division cycle 27, *CRC* colorectal cancer, *CTNNB1* cell division cycle 27, *DKK1-4* Dickkopf WNT signaling pathway inhibitor 1-4, *DNA* deoxyribonucleic acid, *EDNRB* endothelin receptor type B, *ERBB2* erb-b2 receptor tyrosine kinase 2, *ERBB3* erb-b2 receptor tyrosine kinase 3, *FAM123B* (also known as AMER1) APC membrane recruitment protein 1, *FBXW7* F-box and WD repeat domain containing 7, *FZD10* frizzled class receptor 10, *FZD3* frizzled class receptor 3, *GPC6* glypican 6, *IGF1R* insulin-like growth factor 1 receptor, *IGF2* insulin-like growth factor 2, *IRS2* insulin receptor substrate 2, *KIAA1804* (also known as MAP3K21) mitogen-activated protein kinase kinase kinase 21, *KRAS* Kirsten rat sarcoma viral oncogene homolog, *MAP7* microtubule-associated protein 7, *MAPK* mitogen-activated protein kinase, *MIER3* MIER family member 3, *MSH3* mutS homolog 3, *MSH6* mutS homolog 6, *MYO1B* myosin IB, *NRAS* neuroblastoma RAS viral (v-ras) oncogene homolog, *PI3K* phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic, *PIK3CA* phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha, *PIK3R1* phosphoinositide-3-kinase regulatory subunit 1, *PTEN* phosphatase and tensin homolog, *PTPN12* protein tyrosine phosphatase, non-receptor type 12, *SMAD2* SMAD family member 2, *SMAD3* SMAD family member 3, *SMAD4* SMAD family member 4, *SOX9* SRY-box 9, *TCERG1* transcription elongation regulator 1, *TCF7L2* transcription factor 7 like 2, *TGFB2* transforming growth factor beta 2, *TGFBR1* transforming growth factor beta receptor 1, *TGFBR2* transforming growth factor beta receptor 2, *TGF-β* transforming growth factor beta pathway, *TP53* tumor phosphoprotein 53, *TTN* titin, *WNT* Wnt family pathway

challenge is ongoing and the big picture of CRC genetics is not yet established (for further reading, see Guinney et al. 2015).

## 2.3 Oncogenomic Alterations in Colorectal Cancer as Prognostic and Predictive Biomarkers

Recent improvements in CRC survival are attributed to systemic therapy developments in the adjuvant setting based on combined chemotherapies as well as emerging targeted agents for advanced and metastatic disease (Kuipers et al. 2015). Various chemotherapeutic protocols are used in clinical practice as first-line treatment including doublets: FOLFOX [leucovorin + 5-fluorouracil (5-FU) + oxaliplatin], FOLFIRI [leucovorin + 5-FU + irinotecan], CAPEOX [capecitabine + oxaliplatin], and triplets: FOLFOXIRI [leucovorin + 5-fluorouracil + oxaliplatin + irinotecan] (NCCN Guidelines 2019; Van Cutsem et al. 2014). Single-agent approaches are reserved for elderly and frail CRC patients and are based on capecitabine or 5-FU alone or combined with a targeted therapy such as bevacizumab (NCCN Guidelines 2019). Targeted blockade of overexpressed pathways such as EGFR and VEGFR (antiangiogenics and anti-EGFR monoclonal antibodies (cetuximab-Erbitux® or panitumumab-Vectibix®)) showed promising efficacy in a metastatic setting in terms of progression-free survival (PFS) and overall survival (OS) (Kuipers et al. 2015; Battaglin et al. 2017; Sotelo Lezama et al. 2014). However, CRC patients harboring some genetic signatures such as *KRAS* and *BRAF* may have limited benefit from these treatments. Of note, a recent meta-analysis of randomized and controlled trials (RCTs) showed that CRC patients with left-sided wild-type *RAS* status can be considered for anti-EGFR blockade (Holch et al. 2017). In right-sided CRC, adding bevacizumab to standard chemotherapy may be a treatment option (Holch et al. 2017). Remarkably, a very recently published prospective report in *Cancer Cell* demonstrated that tumor sidedness is a promising biomarker with both predictive and prognostic impact in metastatic CRC (Yaeger et al. 2018). The authors sequenced 1134 metastatic CRCs and found significant activating alterations in the WNT pathway in 96% of the tumors. Notably, a shorter survival and enriched tumors with mutated *KRAS*, *BRAF*, *AKT1*, *RNF43*, and *SMAD4* were observed in right-sided tumors compared with the left-sided CRCs that had no mutations in mitogenic pathways and therefore suggesting that they may have two different oncogenic origins (Yaeger et al. 2018).

Genetic testing is an emerging field in oncology practice and a promising advance in the era of personalized therapy (Vakiani 2017; El Bairi et al. 2017b, c; Malapelle et al. 2014; Boutros 2015; Kalia 2015). In addition to their potential diagnostic value as discussed earlier, colorectal oncogenetic alterations are well-established prognostic and predictive biomarkers for therapy response and optimal treatment selection (Vakiani 2017; Malapelle et al. 2014; Sinicrope et al. 2016; Malesci and Laghi 2012; Perincheri and Hui 2015).

### 2.3.1 *RAS* Mutations

RAS proteins are key pleiotropic transduction signals between the membrane receptors such as EGFR and transcriptional factors in the nucleus. Data have accumulated from recent studies suggesting a key role of KRAS signaling in the modulation of the tumor microenvironment by influencing infiltrating immune cells (Dias Carvalho et al. 2018). These signaling components are encoded by three different genes *KRAS*, *NRAS*, and *HRAS* and regulate diverse cell functions encompassing proliferation and cell death (Vakiani 2017). Activating canonical mutations in these oncogenes lead to a constitutive deregulation of the downstream effectors of the EGFR signaling. In CRC, mutations in exon 2 (codons 12 and 13) are the most common (Douillard et al. 2013; Van Cutsem et al. 2015; Vakiani 2017). Moreover, mutations in exons 3 and 4 of *KRAS* and exons 2, 3 and 4 of *NRAS* were also noted in genetic analyses of some pilot clinical trials (Douillard et al. 2013; Van Cutsem et al. 2015). These mutations are excellent predictors of the resistance to the blockade of the upstream receptor by cetuximab or panitumumab alone or combined with chemotherapy as demonstrated by numerous RCTs and meta-analyses (Table 2.3). Previously, an early pilot trial (NCT00113776) demonstrated clearly that wild-type *KRAS* is mandatory for panitumumab activity in metastatic CRC patients (Amado et al. 2008). Data were collected from an open-label phase III trial comparing panitumumab with best supportive care (Amado et al. 2008). PFS was improved in the wild-type group than the mutated group (hazard ratio [HR]: 0.45; 95% CI: 0.34–0.59 vs. HR: 0.99; 95% CI, 0.73–1.36, respectively, $p < 0.0001$). In addition, patients with wild-type status had longer OS and better RR (17 vs. 0% for the mutant group) (Amado et al. 2008). In a similar study, Karapetis et al. analyzed tumor samples collected from 394 advanced CRC patients who were randomized to receive cetuximab plus best supportive care or best supportive care alone (NCT00079066) (Karapetis et al. 2008). Mutated *KRAS* was found in 42.3% of enrolled patients. As expected, cetuximab was found significantly effective in CRC patients bearing wild-type status in terms of PFS (median, 3.7 vs. 1.9 months; HR: 0.40; 95% CI, 0.30–0.54; $p < 0.001$) and OS (median, 9.5 vs. 4.8 months; HR: 0.55; 95% CI, 0.41–0.74; $p < 0.001$) (Karapetis et al. 2008). In 2009, Van Cutsem et al. randomized two groups of 599 CRC patients with unresectable metastases to receive either cetuximab-FOLFIRI or FOLFIRI alone (NCT00154102, CRYSTAL phase III trial) (Van Cutsem et al. 2009). Mutated *KRAS* was confirmed as a powerful predictive biomarker for the cetuximab-FOLFI RI arm efficacy (Van Cutsem et al. 2011). Likewise, randomized phase II OPUS study (cetuximab plus FOLFOX-4) provided similar conclusions regarding the predictive value of this biomarker (Bokemeyer et al. 2011). Moreover, in a large multicenter RCT comparing FOLFOX4 and panitumumab versus FOLFOX4 alone as first-line therapy for metastatic CRC (PRIME study), patients with mutated *KRAS* treated with panitumumab-FOLFOX4 arm had significantly reduced PFS compared with chemotherapy alone (HR: 1.29; 95% CI, 1.04–1.62; $p = 0.02$), and median OS was 15.5 months vs. 19.3 months, respectively (HR: 1.24; 95% CI, 0.98–1.57; $p = 0.068$) (Douillard et al. 2010). Later, these same investigators provided a

**Table 2.3** Predictive meta-analyses of *KRAS, NRAS, BRAF, PIK3CA,* and *PTEN* alterations in colorectal cancer EGFR-based therapy

| Authors/ Year | Journal[a] | *N*[a] | Type of enrolled studies[b] | Number of enrolled patients[c] | Country[d] | Exon | Findings/Conclusions |
|---|---|---|---|---|---|---|---|
| *KRAS* status | | | | | | | |
| Rowland et al. (2016) | Eur J Cancer | 8 | Randomized and controlled trials (RCTs) | 5967 | Australia | 2 | • Efficacy of anti-EGFR therapy does not differ significantly between *KRAS* G13D and other *KRAS* mutations in metastatic colorectal cancer (mCRC) |
| Sorich et al. (2015) | Ann Oncol | 9 | RCTs | 5948 | Australia | 3 and 4 | • No difference in progression-free survival (PFS) or overall survival (OS) benefit was evident between tumors with mutated *KRAS* exon 2 and tumors with the new *KRAS* mutations (exons 3 and 4) |
| Therkildsen et al. (2014) | Acta Oncol | 5 | 4 retrospective studies (RSs) and 1 prospective study (PS) | 867 (total: $n = 2395$) | Denmark | 3 and 4 | • *KRAS* mutations (exons 3 and 4) predict resistance to anti-EGFR therapy<br>• *KRAS* analysis beyond exon 2 must be implemented |
| Chen et al. (2013) | Cancer Chemother Pharmacol | 7 | 5 RSs and 2 PSs | 2802 | China | 2 | • mCRC patients with mutated *KRAS* (codon 13) had a greater clinical response to anti-EGFR-based therapy |
| Mao et al. (2013) | Cancer | 10 | RSs | 1487 | China | 2 | • mCRC patients with mutated *KRAS* p. G13D had a longer PFS and OS and a significantly higher ORR than those with *KRAS* codon 12 mutations |
| Adelstein et al. (2011) | Eur J Cancer | 11 | RCTs | 8924 | Australia | 2 and 3 or NS | • *KRAS* mutations modify the effect of anti-EGFR therapy in mCRC |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Zhang et al. (2011) | Int J Colorectal Dis | 4 | RCTs | 2912 | China | NS | • Mutated *KRAS* patients treated with cetuximab + chemotherapy did not show a significant difference in therapy response (response rate (RR), PFS, and OS) compared with those treated with chemotherapy alone |
| Lin et al. (2011) | Clin Colorectal Cancer | 8 | RCTs | 5325 | USA | NS | • Mutated *KRAS* had a positive likelihood ratio for predicting non-response to anti-EGFR therapy in distant-stage mCRC |
| Qiu et al. (2010) | Eur J Cancer | 22 | 18 RSs and 4 PSs | 2188 | China | 1 and 2 | • Median PFS and OS were significantly shorter in mutant *KRAS* patients<br>• Mutated *KRAS* patients are more likely to have a worse response to anti-EGFR based therapy |
| Linardou et al. (2008) | Lancet Oncol | 8 | 2 PSs and 6 RSs | 817 | Greece | 2 | • This study provides the first meta-analytic and empirical evidence that mutated *KRAS* in mCRC patients is a highly specific negative predictor of response to anti-EGFR therapy |
| *NRAS* status | | | | | | | |
| Sorich et al. (2015) | Ann Oncol | 9 | RCTs | 5948 | Australia | 2–4 | • *NRAS* exons 2, 3, and 4 mutations search must be undertaken before the administration of anti-EGFR therapy |
| Therkildsen et al. (2014) | Acta Oncol | 3 | 2 RSs and 1 PS | 833 (total: $n = 2395$) | Denmark | 2–4 | • Mutated *NRAS* has no significant effect on therapy response, but a significantly decreased PFS and OS |
| *BRAF* status | | | | | | | |
| Pietrantonio et al. (2015) | Eur J Cancer | 9 | RCTs | 463 | Italy | 15 | • Assessment of *BRAF* mutation before initiation of anti-EGFR therapy is supported |

(continued)

**Table 2.3** (continued)

| Authors/ Year | Journal[a] | *N*[a] | Type of enrolled studies[b] | Number of enrolled patients[c] | Country[d] | Exon | Findings/Conclusions |
|---|---|---|---|---|---|---|---|
| Rowland et al. (2015a) | Br J Cancer | 7 | RCTs | 3168 | Australia | 15 | • Evidence is insufficient to definitively state that wt*KRAS*/mutated *BRAF* mCRC patients attain a different treatment benefit from anti-EGFR therapy compared to patients with wt*KRAS*/wt*BRAF* |
| Therkildsen et al. (2014) | Acta Oncol | 17 | 13 RSs, 3 PSs, and 1 retro-prospective | 2079 (total: $n = 2395$) | Denmark | 11 and 15 | • Patients with *BRAF* mutations had a significantly short PFS and OS and had lower objective response rate (ORR) compared to patients with *BRAF* wild-type (wt) tumors |
| Wang et al. (2014) | Chin Med Sci J | 7 | 6 RSs and 1 PS | 1352 | China | 15 | • Mutated *BRAF* V600E is associated with lack of response and worse survival in wt*KRAS* mCRC patients treated with anti-EGFR therapy |
| Yang et al. (2013) | Int J Cancer | 13 | 10 RSs and 3 PSs | 1472 | China | 11 and 15 | • Mutated *BRAF* was associated with shorter PFS, OS, and lower ORR |
| Xu et al. (2013) | J Dig Dis | 19 | 16 RSs and 3 PSs | 2875 | China | 15 | • Mutated *BRAF* is associated with poor response to anti-EGFR therapy and shorter median PFS and OS in mCRC patients |
| Mao et al. (2011) | Mol Biol Rep | 11 | RSs | 1046 | China | 15 | • *BRAF* V600E mutation is associated with poor response in wt*KRAS* mCRC patients treated with anti-EGFR therapy |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *PIK3CA* status | | | | | | | |
| Therkildsen et al. (2014) | Acta Oncol | 7 | 5 RSs and 2 PSs | 716 (total: $n = 2395$) | Denmark | 9 and 20 | • Mutated *PIK3CA* significantly predicted poor ORR. PFS and OS were significantly shorter in comparison with wt status |
| Yang et al. (2013) | Int J Cancer | 5 | 3 PSs and 2 RSs | 748 | China | 9 and 20 | • Mutated *PIK3CA* exon 20 but not exon 9 was associated with shorter PFS, OS, and lower ORR |
| Wu et al. (2013) | J Cancer Res Clin Oncol | 8 | RSs | 839 | China | NS | • *PIK3CA* mutations had a promising predictive value for poor survival in mCRC patients treated with anti-EGFR therapy, particularly in wt*KRAS* patients |
| Mao et al. (2012) | Ann Oncol | 13 | RSs | 576 | China | 9 and 20 | • In mCRC with wt *KRAS,* patients with mutated *PIK3CA* exon 20 had a lower ORR although the combined result was not statistically significant due to the small sample size |
| Loss of *PTEN* expression | | | | | | | |
| Therkildsen et al. (2014) | Acta Oncol | 9 | 8 RSs and 1 PS | 634 (total: $n = 2395$) | Denmark | – | • mCRC patients with *PTEN* loss showed significantly lower ORR, PFS, and OS than those with functional PTEN |
| Yang et al. (2013) | Int J Cancer | 8 | 8 RSs | 591 | China | – | • *PTEN* loss is a predictor of poor response and worse outcomes in mCRC patients with wt*KRAS* treated with anti-EGFR therapy |
| Wang et al. (2012b) | Cancer Chemother Pharmacol | 12 | RSs | 852 | China | – | • *PTEN* loss might be a predictive biomarker of resistance to anti-EGFR therapy in mCRC |

(continued)

**Table 2.3** (continued)

| Authors/ Year | Journal[a] | *N*[a] | Type of enrolled studies[b] | Number of enrolled patients[c] | Country[d] | Exon | Findings/Conclusions |
|---|---|---|---|---|---|---|---|
| Shen et al. (2012) | World J Gastroenterol | 8 | 4 RSs and 4 cohorts | 698 | China | – | • PTEN positivity was associated with better PFS but not with better overall survival OS in CRC patients |

*BRAF* v-raf murine sarcoma viral oncogene homolog B (also known as: B-Raf proto-oncogene, serine/threonine kinase), *EGFR* epidermal growth factor receptor, *KRAS* Kirsten rat sarcoma viral oncogene homolog, *mCRC* metastatic colorectal cancer, *NRAS* neuroblastoma RAS viral (v-ras) oncogene homolog, *NS* not specified in the selected studies, *ORR* objective response rate, *OS* overall survival, *PFS* progression-free survival, *PIK3CA* phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha, *PSs* prospective studies, *PTEN* phosphatase and tensin homolog, *RCTs* randomized and controlled trials, *RR* response rate, *RSs* retrospective studies, *wt* wild type

[a]Number of enrolled studies

[b]In some studies, the retrospective or prospective design was not mentioned (case of proceedings, etc.) and was deduced from other references

[c]Number of enrolled patients in all studies included in the meta-analysis of each gene (retrieved from the Forest plots of the meta-analysis)

[d]Country of the first author

prospective-retrospective analysis of the PRIME study; 639 metastatic CRC patients without mutated *KRAS* exon 2 had results for *BRAF* exon 15, *KRAS* exon 3 or 4, or *NRAS* exons 2–4 (Douillard et al. 2013). Remarkably, these additional mutations predicted a lack of response in patients treated with panitumumab-based therapy (Douillard et al. 2013). A retrospective consortium analysis found similar results for *NRAS* mutations in patients treated with cetuximab in terms of disease control and prediction of therapy response (De Roock et al. 2010). Recently, a post hoc analysis of the CRYSTAL trial investigated the impact on treatment efficacy of other *RAS* mutations other than the traditionally mutated *KRAS* codon 12 or 13 (Van Cutsem et al. 2015). In this subgroup analysis, patients were reanalyzed for other *RAS* mutations (*KRAS* exons 3 and 4, and *NRAS* exons 2, 3, and 4) using digital PCR (BEAMing) (Van Cutsem et al. 2015). *RAS* wild-type patients had more favorable OS and ORR from the combination of cetuximab with FOLFIRI compared to patients with other *RAS* mutations (Van Cutsem et al. 2015). However, the enrolled group with other *RAS* mutations was relatively small, and therefore no definitive conclusions could be drawn. Interestingly, a meta-analytic combination of these studies provided strong evidence for using these mutations in clinical practice as predictive biomarkers for a successful therapy based on anti-EGFR monoclonal antibodies (Table 2.3). Based on this large body of evidence, the current NCCN guidelines for CRC management require the determination of *RAS* status for patients being considered for an anti-EGFR-based therapy (NCCN 2019). Based on an extensive critical assessment of the current data on this topic, most recent collaborative guidelines from the American Society for Clinical Pathology (ASCP), College of American Pathologists (CAP), Association for Molecular Pathology (AMP), and the American Society of Clinical Oncology (ASCO) recommend the molecular testing in the *RAS* genes (*KRAS* and *NRAS*: exons 2, 3, and 4) as they provide actionable information as biomarkers for predicting therapy response to targeted anti-EGFR treatments (strength of evidence (SE): convincing/adequate) (Sepulveda et al. 2017). Accordingly, both panitumumab and cetuximab should only be prescribed for metastatic CRC patients who are wild-type for all known activating *RAS* mutations until this time (see meta-analysis of RCTs by Sorich et al. 2015).

### 2.3.2 *BRAF* Mutations

*BRAF* encodes for a mitogenic serine/threonine kinase involved in the EGFR pathway via MAPK signals (Hertzman Johansson and Egyhazi Brage 2014). RAF proteins are the first effectors of RAS GTPase signaling cascade. Mutations by gain of function in $BRAF^{V600E}$ conferring a constitutive kinase activity are present in about 10–15% of all CRC patients (Barras 2015; Vakiani 2017). In CRC, prognostic value of mutated *BRAF* has been evaluated in various interventional clinical trials (Van Cutsem et al. 2011; Ogino et al. 2012; Bokemeyer et al. 2012; Schirripa et al. 2015; Kaczirek et al. 2015). Recent evidence supports the role of *BRAF* status more in prognosis than its predictive value in therapy response (Vakiani 2017; Roth et al. 2010; Bokemeyer et al. 2012). A recent meta-analysis of nine RCTs reported that

adding an anti-EGFR treatment in patients with mutated *BRAF* did not improve PFS, OS, and ORR (HR: 0.88; 95% CI: 0.67–1.14; $p = 0.33$; HR: 0.91; 95% CI: 0.62–1.34; $p = 0.63$; RR: 1.31; 95% CI: 0.83–2.08, $p = 0.25$, respectively) (Pietrantonio et al. 2015). Further, results from other meta-analyses tend to draw same conclusions (Table 2.3). On the other hand, Rowland et al. conducted a meta-analysis of seven RCTs and found contradictory conclusions (Rowland et al. 2015a). This study stated that the observed differential effects of anti-EGFR-based therapy in mutated *BRAF* on OS may be due only to chance since the interaction test was not statistically significant ($p = 0.43$) (Rowland et al. 2015a). In a letter by Pietrantonio's team (Cremolini et al. 2015), analyses in enrolled RCTs of the Rowland's meta-analysis were retrospective with an unplanned evaluation of patients' subgroups. Moreover, it should also be noted that low incidence of mutated *BRAF* in enrolled patients is a major concern that definitely underpowers the statistical analysis in this meta-analytic investigation (Cremolini et al. 2015). In their reply, Rowland et al. responded by the fact that such concerns can be related to the post hoc nature of many subgroup analyses and the high risk of false positives with multiple hypotheses testing (Rowland et al. 2015b).

Importantly, from a pharmaco-economic point of view, an analysis by Behl et al. addressed the problem of cost-effectiveness of molecular testing and found that testing for *BRAF* saves $1023 per patient in addition to $7500 for *KRAS* compared with anti-EGFR without screening (Behl et al. 2012). However, to date, ASCP, CAP, AMP, and ASCO collaborative guidelines do not recommend *BRAF* testing for response to anti-EGFR therapy in CRC (SE: insufficient) but support it for only diagnosis and prognostic stratification (Sepulveda et al. 2017). In conclusion, accurate and definitive evidence about this unresolved problem is to be demonstrated by ongoing and future clinical trials especially those investigating BRAF and MEK inhibitors such as vemurafenib, dabrafenib, and trametinib (prognostic value of *BRAF* mutations in first-line, second-line, and real-world is discussed by Strickler et al. 2017). Interestingly, the phase 3 BEACON CRC trial (NCT02928224) enrolled 665 patients with $BRAF^{V600E}$ mutant metastatic CRC and evaluated the use of a triplet targeted therapy (RAF, MEK, and EGFR inhibition) (Van Cutsem et al. 2019). The safety lead-in findings were recently published and showed manageable toxicity profile to start the randomized portion of the study (Van Cutsem et al. 2019). Moreover, the prespecified interim analysis demonstrated a gain in OS (HR: 0.52; 95% CI: 0.39–0.70; $p < 0.001$) and response rate in the arm combining encorafenib, cetuximab, and binimetinib as compared to the arm using the standard cetuximab and irinotecan or cetuximab and FOLFIRI in metastatic CRC with mutated *BRAF* (Kopetz et al. 2019; Huijberts et al. 2020). Thus, *BRAF* shows again actionable information as a potential predictive and targetable mutation in this setting.

### 2.3.3 *PIK3CA* Mutations

About 10–18% of CRC patients harbor downstream gain-of-function *PIK3CA* mutations usually in exons 9 and 20 with an increased risk of oncogenic

transformation ability (Sepulveda et al. 2017). In general, reports investigating the prognostic value of these alterations indicated poor outcomes in CRC patients (Ogino et al. 2009; De Roock et al. 2010; Liao et al. 2012; Karapetis et al. 2014). To date, several meta-analytic approaches explored their potential as a predictive biomarker for anti-EGFR therapy and survival (Table 2.3). In a large meta-analysis enrolling 20 studies, Yang et al. found that mutated *PIK3CA* exon 20 but not exon 9 is associated with shorter PFS, OS, and lower ORR (Yang et al. 2013). These results were confirmed recently by another meta-analysis who found that mutated *PIK3CA* significantly predicted poor ORR compared to wild-type status (Therkildsen et al. 2014). Further, in metastatic CRC with wild-type *KRAS*, patients with mutated PIK3CA exon 20 had a lower ORR although the combined result was not statistically significant due to the small sample size (Mao et al. 2012). However, these findings need to be interpreted with caution because of the risk of bias in enrolled studies, the potential conflicts of the panelists, as well as the small number of identified mutations. Selection of CRC patients to benefit from anti-EGFR therapy based on *PIK3CA* mutational status is not recommended by the ASCP, CAP, AMP, and ASCO collaborative guidelines and must be provided only in the context of clinical trials (SE: insufficient) (Sepulveda et al. 2017).

### 2.3.4 Microsatellite Instability

Incorporation of MSI testing in the clinical management of CRC is a recent advance. In addition to its diagnostic potential in Lynch syndrome, MSI showed robust evidence as prognostic and predictive biomarker for some clinical settings especially for adjuvant chemotherapy and emerging immunotherapy (Kawakami et al. 2015; Westdorp et al. 2016). Several years ago, first reports highlighted the impact of MSI on the benefit from 5-FU-based chemotherapy (Ribic et al. 2003; Arnold et al. 2003; Tajima et al. 2004). Later, Sargent et al. examined MMR status as a predictive biomarker for 5-FU-based therapy in CRC patients with stages II and III ($n = 457$) (Sargent et al. 2010). Overall, patients with defective MMR status treated with 5-FU adjuvant therapy had a worse disease-free survival (DFS) (HR: 1.10; 95% CI, 0.42–2.91; $p = 0.85$) and reduced OS (HR: 2.95; 95% CI, 1.02–8.54; $p = 0.04$) compared with patients receiving surgery alone (Sargent et al. 2010). In a large study, Sinicrope et al. included stage II and III CRC patients ($n = 2141$) who were treated in randomized trials using 5-FU-based therapy; tumors were analyzed for MSI by immunohistochemistry and PCR-based assay (Sinicrope et al. 2011). Contrary to the previous results, Sinicrope et al. reported that defective MMR was associated with reduced 5-year recurrence rates and fewer distant recurrences (Sinicrope et al. 2011). In addition, patients with stage III CRC with defective MMR who were treated with 5-FU-based therapy had reduced distant recurrence compared with patients with proficient MMR status (11 vs. 29%; $p = 0.011$) (Sinicrope et al. 2011). However, this question remains complex and elusive and some meta-analyses combined many study results to find strong evidence. In this perspective, two meta-analyses (in total 38 studies and 16,472 CRC patients)

confirmed the association between MSI-high and favorable prognosis and prediction of non-response to 5-FU-based therapy (Des Guetz et al. 2009; Guastadisegni et al. 2010). In contrast to the previous combined results, Webber et al. enrolled 16 studies (9212 patients) assessing the association between MSI, DFS, and OS; meta-analysis of 14 eligible studies found that there is no significant difference in the effect of 5-FU-based treatment based on MSI status (Webber et al. 2015). Again, these studies must be interpreted carefully because of the possible risk of conflicts of interest and pitfalls due to non-randomized comparisons and biases. In their critical literature review, the ASCP, CAP, AMP, and ASCO collaborative guidelines recommend MSI testing to assess the risk for Lynch syndrome and/or prognostic stratification but not as a predictive biomarker for adjuvant therapy (SE: adequate/inadequate) (Sepulveda et al. 2017). Interestingly, a recent phase II proof-of-concept trial showed that MMR status is a potential predictive biomarker of clinical response to immune-checkpoint blockade with pembrolizumab (Le et al. 2015). More recently, a report in *Science* found that neoantigens in CRC patients with MMR deficiency make them more sensitive to PD-1 blockade which expands the value of MSI as a genetic biomarker for other emerging targeted therapies (Le et al. 2017). Moreover, metastatic CRC patients treated with bevacizumab and with MSI-high tumors showed improved OS as compared to those in the cetuximab arm (HR: 0.13, 95% CI: 0.06–0.30; $p < 0.001$) (Innocenti et al. 2019). Therefore, additional human trials are awaited to confirm these findings.

### 2.3.5 Loss of *PTEN* Expression

Predictive and prognostic value of *PTEN* expression loss in CRC has been evaluated in few published works and is still controversial (Molinari and Frattini 2013). On the one hand, some studies showed that loss of *PTEN* expression was found correlated strongly with a later stage of CRC, liver metastasis, and 5-year survival (Nassif et al. 2004; Sawai et al. 2008; Atreya et al. 2013). On the other hand, other studies did not show significant prognostic information regarding *PTEN* expression loss (Eklöf et al. 2013; Price et al. 2013). Predictive value of this biomarker in anti-EGFR therapy was found negatively associated with response (Negri et al. 2010; Frattini et al. 2007; Perrone et al. 2009). In addition, discordant findings were also noted in other studies and failed to demonstrate strong association between *PTEN* expression loss and prediction of response to anti-EGFR therapy (Ulivi et al. 2012; Tol et al. 2010). Four meta-analyses (in total 38 studies and 2241 CRC patients) addressed this issue (Therkildsen et al. 2014; Yang et al. 2013; Wang et al. 2012b) and supported the fact that *PTEN* loss predicts resistance to anti-EGFR monoclonal antibodies (Table 2.3). However, *PTEN* analysis by immunohistochemistry or gene deletion detection by FISH (fluorescence in situ hybridization) is not recommended according to the ASCP, CAP, AMP, and ASCO collaborative guidelines except for patients being programmed for clinical trials (SE: insufficient) (Sepulveda et al. 2017).

### 2.3.6 *HER2* (*ERBB2*) Alterations

HER2 is a tyrosine kinase receptor with similar functions to the EGFR signaling pathway (Appert-Collin et al. 2015). HER2 blockade by monoclonal antibodies (e.g., trastuzumab and pertuzumab) and tyrosine kinase inhibitors (TKIs) such as lapatinib and neratinib provides promising therapeutic activities in various epithelial cancers including gastrointestinal (Hsu and Hung 2016; Mar et al. 2015; Buza et al. 2014; Boku 2014; Oh and Bang 2016). *ERBB2* amplifications are found in 4% of metastatic CRC cases (Yaeger et al. 2018), are more prevalent in *KRAS*/*BRAF* wild-type tumors (Sartore-Bianchi et al. 2016; Herreros-Villanueva et al. 2011), and are correlated with protein overexpression of HER2 (Vakiani 2017). HER2 status can be determined reliably by using standard immunohistochemistry, FISH, and NGS techniques (Ross et al. 2017; Valtorta et al. 2015). Importantly, two recent meta-analyses demonstrated that there is no significant relation between HER2 expression and poor prognosis in CRC patients (Wu et al. 2014, 2015). *ERBB2* amplification was found to drive de novo and acquired resistance to EGFR inhibition by cetuximab (Yonesaka et al. 2011; Bertotti et al. 2015). Promisingly, Kavuri et al. showed that dual targeting of HER2 by trastuzumab plus TKIs produced regression of colorectal tumors in in vivo models (Kavuri et al. 2015). HER2 alterations have recently gained attention as a druggable pathway in CRC (The Cancer Genome Atlas Network 2012; Ingold Heppner et al. 2014) and are being targeted in several ongoing phase I and II trials (https://www.clinicaltrials.gov/ct2/results?cond=Colorectal+Cancer&term=HER2&cntry=&state=&city=&dist). In this direction, remarkable results from HERACLES phase II trial demonstrated that HER2 blockade with a combination of trastuzumab and lapatinib is active and well tolerated in treatment-refractory metastatic CRC patients with HER2-positive status (Sartore-Bianchi et al. 2016). Later, the combination of pertuzumab and trastuzumab-emtansine was added to the HERACLES trial. Pertuzumab combined with trastuzumab was evaluated recently by the phase IIa "My Pathway" multibasket trial (NCT02091141) and demonstrated tolerable toxicity profile in heavily pretreated, HER2-amplified metastatic CRC (Meric-Bernstam et al. 2019). At the present time, NCCN guidelines do not recommend HER2 testing for prognostication and therapy selection until confirmatory evidence from ongoing studies is available (NCCN guidelines 2019). Remarkably, these advances in molecular profiling were achieved because of the newly developed NGS technology which allowed a deep, rapid, and efficient analysis of the CRC genomic alterations.

## 2.4 Conclusion

Management of CRC is still evolving especially with the several positive clinical trials published every year. Notably, CRC is one of cancers that have benefited the most from the genetic advances of the last decade. Genetic biomarkers are currently used in clinical decision-making when delivering care to CRC patients particularly with the emergence of NGS technologies and targeted therapies and are no longer a

concept but a reality for routine use. However, tailoring CRC management needs more clinical trials with basket designs and parallel assessment of predictive biomarkers; which are needed to provide additional evidence for these therapeutic advances (see Boxes 2.1 and 2.2 for recommended reading).

**Box 2.1 Recommended Reading for English-Speaking Readers from Highly Accessed Medline-Indexed Journals**

| | |
|---|---|
| Li et al. *Mismatch Repair and Colon Cancer: Mechanisms and Therapies Explored*. Trends Mol Med. 2016;22(4):274-289. | doi:https://doi.org/10.1016/j.molmed.2016.02.003 |
| Valle L. *Recent Discoveries in the Genetics of Familial Colorectal Cancer and Polyposis*. Clin Gastroenterol Hepatol. 2017;15(6):809-819. | doi:https://doi.org/10.1016/j.cgh.2016.09.148 |
| Cox AD, et al. *Drugging the undruggable RAS: Mission possible?*. Nat Rev Drug Discov. 2014;13(11):828-51. | doi:https://doi.org/10.1038/nrd4389 |
| Cerretelli G, et al. *Molecular Pathology of Lynch Syndrome* [published online ahead of print, 2020 Mar 6]. J Pathol. 2020;https://doi.org/10.1002/path.5422. | doi:https://doi.org/10.1002/path.5422 |
| Lopes G, et al. *Early Detection for Colorectal Cancer: ASCO Resource-Stratified Guideline*. J Glob Oncol. 2019;5:1–22. | doi:https://doi.org/10.1200/JGO.18.00213 |
| Grolleman JE, et al. *Somatic mutational signatures in polyposis and colorectal cancer*. Mol Aspects Med. 2019;S0098-2997(19)30007-X. | doi:https://doi.org/10.1016/j.mam.2019.05.002 |
| Shen H, Laird PW. *Interplay between the cancer genome and epigenome*. Cell. 2013;153(1):38-55. | doi:https://doi.org/10.1016/j.cell.2013.03.008 |
| Dancey JE, et al. *The genetic basis for cancer treatment decisions*. Cell. 2012;148(3):409–420. | doi:https://doi.org/10.1016/j.cell.2012.01.014 |
| Olivera G, et al. *Colorectal cancer: pharmacogenetics support for the correct drug prescription*. Pharmacogenomics. 2019;20(10):741–763. | doi:https://doi.org/10.2217/pgs-2019-0041 |
| Sandhu J, et al. *Systemic treatment for metastatic colorectal cancer in the era of precision medicine*. J Surg Oncol. 2019;119(5):564–582. | doi:https://doi.org/10.1002/jso.25421 |
| Grolleman JE, et al. *Somatic mutational signatures in polyposis and colorectal cancer*. Mol Aspects Med. 2019;S0098-2997(19)30007-X. | doi:https://doi.org/10.1016/j.mam.2019.05.002 |
| Ried T, et al. *The landscape of genomic copy number alterations in colorectal cancer and their consequences on gene expression levels and disease outcome*. Mol Aspects Med. 2019;S0098-2997(19)30035-4. | doi:https://doi.org/10.1016/j.mam.2019.07.007 |
| Dienstmann R, et al. *Consensus molecular subtypes and the evolution of precision medicine in colorectal cancer*. Nat Rev Cancer. 2017;17(2):79–92. | doi:https://doi.org/10.1038/nrc.2016.126 |
| Kraus VB. *Biomarkers as drug development tools: discovery, validation, qualification and use*. Nat Rev Rheumatol. 2018;14(6):354-362. | doi: https://doi.org/10.1038/s41584-018-0005-9. |

(continued)

**Box 2.1** (continued)

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|---|---|
| Cree IA. *Progress and potential of RAS mutation detection for diagnostics and companion diagnostics*. Expert Rev Mol Diagn. 2016;16(10):1067-1072. | doi:https://doi.org/10.1080/14737159.2016.1221345 |

## Box 2.2 Recommended Reading for Non-English-Speaking Readers from Highly Accessed Medline-Indexed Journals

| | |
|---|---|
| Rau TT, et al. *Hereditäres Dickdarmkarzinom: Ein Update zu Genetik und Entitäten aus differenzialdiagnostischer Sicht*. Pathologe. 2017;38 (3):156–163. | doi:https://doi.org/10.1007/s00292-017-0294-9 |
| Neumann JH. *Prognostische Biomarker für das metastasierte kolorektale Karzinom*. Pathologe. 2016;37(Suppl 2):180–185. | doi:https://doi.org/10.1007/s00292-016-0204-6 |
| Bouchez C, et al. *Traitement des autres tumeurs solides métastatiques MSI/dMMR*. Bull Cancer. 2019;106(2):143–150. | doi:https://doi.org/10.1016/j.bulcan.2019.01.008 |
| Buecher B, et al. *Syndrome CMMRD (déficience constitutionnelle des gènes MMR): bases génétiques et aspects cliniques*. Bull Cancer. 2019;106 (2):162–172. | doi:https://doi.org/10.1016/j.bulcan.2018.10.008 |
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| Dreyer C, et al. *Nouvelles classifications moléculaires du cancer colorectal, du cancer du pancréas et du cancer de l'estomac: vers un traitement à la carte?*. Bull Cancer. 2016;103 (7-8):643–650. | doi:https://doi.org/10.1016/j.bulcan.2016.05.007 |
| Lino-Silva LS, et al. *Clasificación Molecular Del Carcinoma De Colon Y Recto. Una Revisión Corta*. Gac Med Mex. 2018;154(5):598–604. | doi:https://doi.org/10.24875/GMM.18003411 |
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| Moreira L. *Novedades en el cáncer colorrectal hereditario*. Gastroenterol Hepatol. 2015;38 Suppl 1:78–85. | doi:https://doi.org/10.1016/S0210-5705(15)30023-6 |
| Chen ZY, et al. 结直肠癌分子分型研究进展. Zhonghua Zhong Liu Za Zhi. 2017;39(9):641–645. | doi:https://doi.org/10.3760/cma.j.issn.0253-3766.2017.09.001 |
| Pan T, et al. 遗传性结直肠癌的基因诊断及治疗进展. Zhang SZ. Zhonghua Zhong Liu Za Zhi. 2013;35 (10):721–725. | doi:https://doi.org/10.3760/cma.j.issn.0253-3766.2013.10.001 |

(continued)

**Box 2.2** (continued)

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| Jeon SY, et al. *대장암의 분자 분류와 임상 적용*. Korean J Gastroenterol. 2016;68(6):297–302. | doi:https://doi.org/10.4166/kjg.2016.68.6.297 |

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# The Arrival of Next-Generation Sequencing: An Overview of Current Technologies

3

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**Abstract**

Over the past decade, substantial progress has been achieved in understanding the molecular mechanisms of carcinogenesis, and several hallmarks defining cancer have been established. These advances have markedly impacted translational research and clinical practice following the arrival of the next-generation sequencing (NGS) technology. This innovative revolution in molecular biology has enabled a rapid interrogation of the cancer genomes even using small quantities of nucleic acids. In this chapter, we describe the advantages and limitations of current NGS platforms including those using sequencing by synthesis, sequencing by ligation, and real-time sequencing, as well as their significant impact in molecular oncology.

**Keywords**

Next-generation sequencing · Sequencing by ligation · Sequencing by synthesis · Real-time sequencing · Cancer genomes

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## 3.1 Introduction

Historically, after the discovery of the DNA structure in 1953 by molecular biologists James Watson and Francis Crick (Watson and Crick 1953) and its sequencing based on autoradiography visualization in 1977 by Sanger et al. (1977) and by Maxam and Gilbert (Maxam and Gilbert 1977), major advances in molecular biology have allowed a better structure and function elucidation of this "magic" molecule. Later, around the 1990s, the first slab gel-based sequencer [ABI PRISM® 3700 DNA Analyzer (Thermo Fisher Scientific, Waltham, MA, USA)] was made available in parallel with the launch of the human genome sequencing project which was declared complete in 2003. Since then, a rapid and extraordinary evolution of this area has allowed more sophisticated, scalable, faster, and cheaper technologies for genome sequencing with a significant increase in fees related to "big data" management based on bioinformatic pipelines and associated errors. Following this after-Sanger era, Roche 454's pyrosequencing system was the first marketed of the NGS platforms launched in 2005 based on light detection of pyrophosphate release in addition to QIAGEN® PyroMark Q series (Margulies et al. 2005; Müllauer 2017; Harrington et al. 2013). Compared to the old classical sequencing methods, NGS enables a simultaneously and massively increased sequencing rate ranging from few gigabases per run to 6000 gigabases and therefore a possible human genome sequencing within 1 week with only 999 US dollars according to Veritas® genomic company (Müllauer, 2017; Goodwin et al. 2016; https://www.veritasgenetics.com/why-are-we-here). Current NGS is categorized into (1) systems that use sequencing by synthesis chemistry [Illumina® platforms (Illumina®, San Diego, CA, USA), Ion Torrent® platforms (Thermo Fisher Scientific, Waltham, MA, USA), QIAGEN GeneReader® (QIAGEN, Hilden, Germany), Roche® Sequencing platforms (Roche, Pleasanton, CA, USA)] and (2) systems that use sequencing by ligation [SOLiD® (Thermo Fisher, Waltham, MA, USA) and BGISEQ-500® (BGI (MGI) Tech, Shenzhen, China)] allowing short-read sequencing approaches (for review, see Goodwin et al. 2016). On the other hand, further recent technologies [Pacific BioSciences® platforms (PACBIO®, California, USA) and 10X Genomics® platforms (10X Genomics, Pleasanton, CA, USA)] enable a long read and real-time sequencing advantages (Goodwin et al. 2016). Interestingly, novel "lab-on-a-chip" technologies such as the freshly introduced IBM® DNA Transistor (IBM®, Armonk, New York, USA) and Oxford Nanopore Technologies (MinION, PromethION, SmidgION platforms; Oxford Nanopore Technologies®, Oxford Science Park, UK) are revolutionizing this field beyond the current next-generation sequencers and enable genome sequencing in real-time conditions (Yang and Jiang 2017; Lu et al. 2016). NGS ranges from the whole-genome sequencing analyzing the totality of human genome to targeted exome sequencing and finally to focused single genetic alteration assays. Most of NGS technologies are still for research use only, but recently, some platforms have been validated and gained approval by the FDA for marketing and routine laboratory use.

## 3.2 Sequencing by Synthesis Platforms

### 3.2.1 Pyrosequencing Systems (Roche® and QIAGEN® PyroMark)

Pyrosequencing principle (Fig. 3.1) is based on single-nucleotide addition methods that quantify the liberated inorganic pyrophosphate (PPi) after incorporation of a nucleic base using a cascade of enzymatic reactions that produces detectable bioluminescence signals (Metzker 2010; Ronaghi et al. 1998). Instead of Sanger sequencing which needs addition of complementary nucleotides all together at the same time into the reaction medium, pyrosequencing incorporates sequentially each known deoxyribonucleotide triphosphate (dNTP) in the elongation single-stranded amplicon by DNA polymerase. A PPi is therefore released and captured by an ATP sulfurylase to produce an ATP molecule which in turn is coupled to a luciferin to generate an oxyluciferin and light signals by luciferase-mediated conversion. An apyrase is added to the reaction wells to degrade the excess of dNTPs, and a camera called charge-coupled device (CCD) enables high-resolution and sensitive detection of generated signals. Of note, recorded light peaks and intensity are proportional to the number of incorporated nucleotides and reveal DNA sequences using different programs (Fig. 3.1b). Before performing pyrosequencing using Roche® 454 platform, template preparation and amplification are required using a microfluidic emulsion PCR (EmPCR) technology that has the advantage to avoid loss of DNA

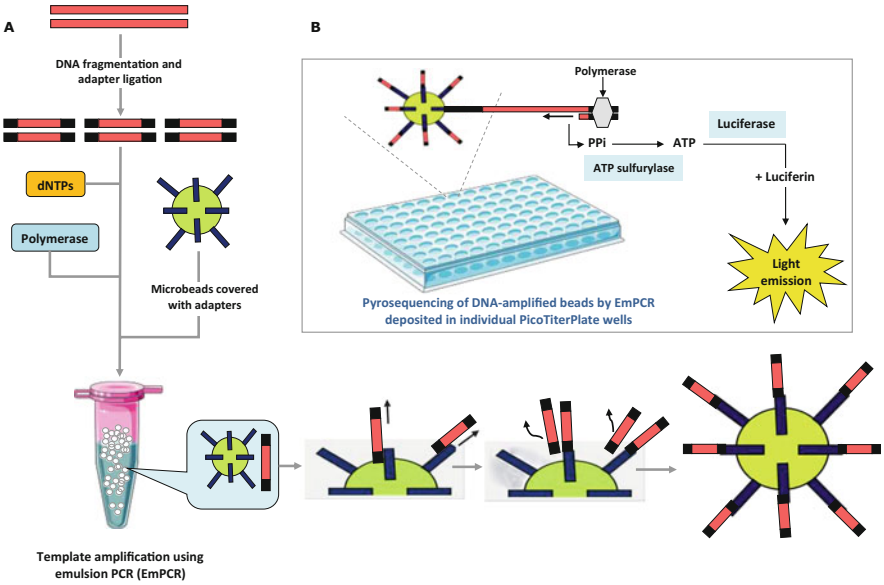


**Fig. 3.1** Simplified diagram of (**a**) emulsion PCR and (**b**) pyrosequencing workflow. For comments, see text. *EmPCR* emulsion polymerase chain reaction, *DNA* deoxyribonucleic acid, *dNTPs* deoxynucleotides, *ATP* adenosine triphosphate, *PPi* pyrophosphate

sequences (for protocol review, see Kanagal-Shamanna 2016). In EmPCR (Fig. 3.1a), first, DNA templates are fragmented by sonication (or other methods), ligated to adapters and denatured followed by a capture in water-in-oil droplets. Each droplet contains DNA template with adapters, complementary adapters loaded on beads, primers, polymerase, and dNTPs. After amplification, millions of clonally amplified beads are placed and arrayed in PicoTiterPlate (PTP) microwells where massively parallel pyrosequencing reactions are performed (Metzker 2010; Goodwin et al. 2016). Despite their fast run times and improved read lengths (Roche® GS FLX Titanium and GS Junior), pyrosequencing machines had high error rates for sequencing homopolymer repeats and high reagent costs as well as difficulties in genome assembly. In 2013, Roche® discontinued its 454-based NGS platforms because of the arrival of highly competitive and coming of age technologies from Illumina® and Ion Torrent® (https://www.fiercebiotech.com/medical-devices/roche-to-close-454-life-sciences-as-it-reduces-gene-sequencing-focus—accessed: 11/05/2018).

### 3.2.2 Illumina® Platforms

So far, Illumina is dominating the market of short-read NGS platforms as a result of its impressive high-throughput sequencing technology and low cost per base (van Dijk et al. 2014). The first NGS platform from Illumina (Genome Analyzer) was launched in 2006 by Solexa (acquired by Illumina one year later) allowing 1 gigabase/run (https://emea.illumina.com/science/technology/next-generation-sequencing/illumina-sequencing-history.html—accessed 18-05-2018). The foundation of Illumina instruments is based on sequencing by synthesis (base-by-base) technology using fluorescently labeled nucleotides (Fig. 3.2). In the first step, DNA is fragmented and ligated to adapters and bound to a solid support (glass flow cell) that contains immobilized primers (two types of oligos, forward and reverse) (Fig. 3.2a, b). The free end of DNA fragments interacts with close oligos, therefore creating bridges, and a clonal amplification PCR is used to generate the second strand. Finally, the bridge is denatured to form single-stranded DNA, the template is washed to remove reverse strands, and the process is repeated over again. In the second step, four differently labeled, fluorescent, and cleavable reversible terminator dNTPs (blockade of their 3′-OH group to prevent elongation) and DNA polymerase are added to the reaction (Guo et al. 2008; Goodwin et al. 2016). Every nucleotide is incorporated one by one into the elongating strand, unbound dNTPs are washed away, and a CCD camera is used to scan and identify which nucleotide is added and another cycle is repeated (Goodwin et al. 2016) (Fig. 3.2c). Illumina developed, refined, and optimized several NGS systems including MiniSeq series, MiSeq series, HiSeq series, HiSeq X series, NextSeq series, and the recently released NovaSeq 600 system that enable a tremendous increase in throughput and generate multiple terabases/run. Illumina MiSeq is designed as a personal sequencer with low run time and is adapted to small genomes. Illumina MiSeq seems to have superior position for metagenomic sequencing and molecular diagnostics laboratory. Moreover, Illumina

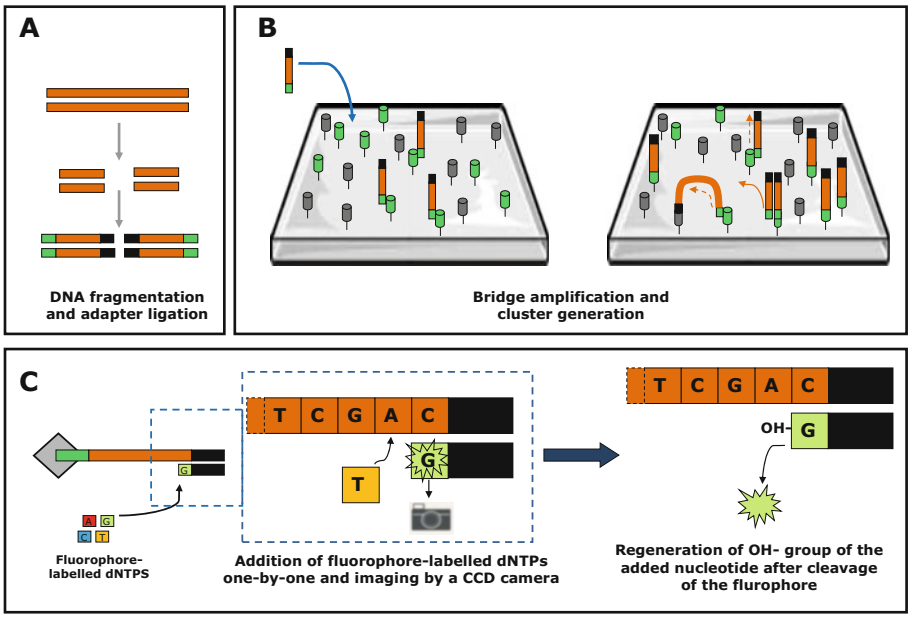


**Fig. 3.2** Principle of Illumina sequencing: (**a**) template preparation, (**b**) amplification, and (**c**) sequencing. For comments, see text

HiSeq series are widely used for high-throughput applications such as large whole-genome sequencing and are more adapted to research use only. Substitution errors across Illumina platforms are the most frequent and are below 1%. In addition, Illumina technology has reduced homopolymer errors compared to other NGS systems using single-nucleotide addition strategies.

### 3.2.3 Thermo Fisher Ion Torrent® Platforms

Ion Torrent® systems share sequencing by synthesis strategy used by other platforms such as pyrosequencing and employ a unique pH-mediated non-optical sequencing (Rothberg et al. 2011). Similar to pyrosequencing, Ion Torrent® uses EmPCR to prepare templates (Fig. 3.1a). DNA-amplified beads are incubated in microwells where sequencing takes place. Nucleotides are added into the reaction one species at a time, and if the dNTP incorporated in the elongation strand is complementary, hydrogen ions ($H^+$) are released and induce pH changes which are detected by ion sensors [CMOS (complementary metal-oxide semiconductor) and ISFET (ion-sensitive field-effect transistor)] placed in the microwells and converted to voltage signals; the residual dNTPs are washed away and another cycle begins (Fig. 3.3). Basically, a voltage signal is proportional to the number of sequential dNTPs added to the elongating strand. Moreover, DNA templates may have homopolymer repeats; thus, multiple dNTPs are added in a distinct cycle and a strong

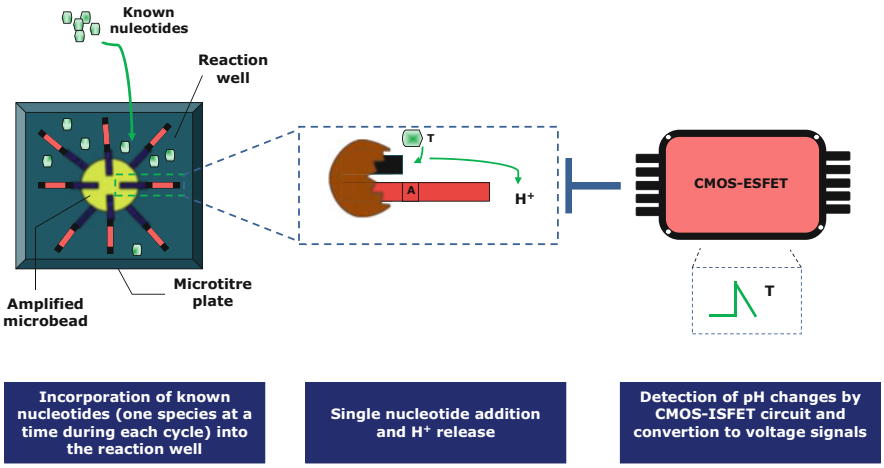


**Fig. 3.3** Principle of Ion Torrent sequencing. For comments, see text. *CMOS* complementary metal-oxide semiconductor, *ISFET* ion-sensitive field-effect transistor

voltage signal is then detected which may limit the strength of this NGS by increasing the error rates (especially indels). However, this non-optical NGS has the advantage to distinguish between incorporated dNTPs during sequencing cycles and therefore enables fast runs and reduces reagents costs. Ion Torrent has marketed two platforms: Ion Torrent PGM which delivers 400 bp of read lengths and 2–7 h run time and Ion Proton system with a read length of 200 bp and a run time between 2 and 4 h. Ion Torrent PGM seems to be the best choice for affordable targeted sequencing panels (Lupini et al. 2015; Haley et al. 2015; Malapelle et al. 2015; Algars et al. 2017) compared to Ion Proton that is more practical for exome and transcriptomic sequencing (Brown et al. 2017).

### 3.2.4 QIAGEN® GeneReader

QIAGEN® introduced its all-in-one NGS system named GeneReader in 2015 (Karow 2015). The GeneReader was developed to perform all the sequencing steps from nucleic acid extraction and clonal amplification using the QIAcube system until data analysis and interpretation workflow. Template enrichment during the preparation phase uses EmPCR as the one used by Roche® pyrosequencing, SOLiD®, and Ion Torrent® platforms. Typically, the GeneReader sequences incorporated fluorescent nucleotides by Illumina platforms and detects signals with imaging by TIRF (total internal reflection fluorescence) microscopy using laser channels (Goodwin et al. 2016) (Fig. 3.4). Sequencing of DNA from FFPE samples from CRC subjects using this NGS system was recently validated with reference to PCR, pyrosequencing, and Illumina MiSeq (Darwanto et al. 2017). Until this time, the GeneReader is intended for cancer clinical research use only.

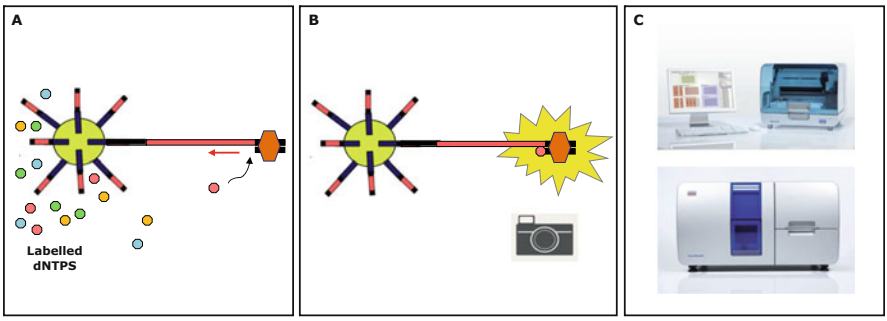


**Fig. 3.4** Principle of Qiagen GeneReader platform: (**a**) addition of fluorophore-labeled dNTPs to hybridize with the complementary strand, (**b**) after the incorporation of fluorophore-labeled dNTP and the cleavage of the fluorophore to regenerate the OH group, the unit is imaged using four laser channels and another cycle begins, and (**c**) top: the QIAcube system, bottom: the Qiagen® GeneReader platform (reused with permission from Qiagen®)

## 3.3 Sequencing by Ligation Platforms

### 3.3.1 Thermo Fisher SOLiD®

SOLiD (Sequencing by Oligonucleotide Ligation and Detection) NGS system was launched by Applied Biosystems Inc. in 2007 (purchased later by Thermo Fisher®) and is based on the use of two-base color encoding and sequencing by ligation strategies (Goodwin et al. 2016; Valouev et al. 2008) allowing a maximum read length of 75 bp (Goodwin et al. 2016). Following DNA amplification generated by EmPCR, 3′-modified beads are deposited to be covalently attached in the surface of the flowchips (glass slides). In each flowchip, a sequence of bases (anchor primer) binds to the adapter and probes containing two first known labeled nucleotides attached to six other bases with a fluorophore hybridized to the strand template using a DNA ligase and the complex is imaged (Goodwin et al. 2016; Meldrum et al. 2011; Shendure et al. 2005) (Fig. 3.5). After this step, cleavage of the fluorophore is performed together with three bases of the probe, and another round of ligation, imaging, and cleavage is completed to recognize two out of every five nucleotides (probe extension). Finally, other sequencing cycles using this time progressive offset primers ($n - 1$, one base shifted) to decode the rest of the strand and therefore allowing an accurate double-sequencing strategy. However, substitution errors and difficulties in sequencing palindromic regions are the drawbacks of this technology (Huang et al. 2012). SOLiD short-read NGS platforms were discontinued as of May 1, 2016, and are no longer available for sale (https://www.thermofisher.com/content/dam/LifeTech/Documents/PDFs/5500_DiscontinuanceLetter_November2015.pdf—accessed 22-05-2018).

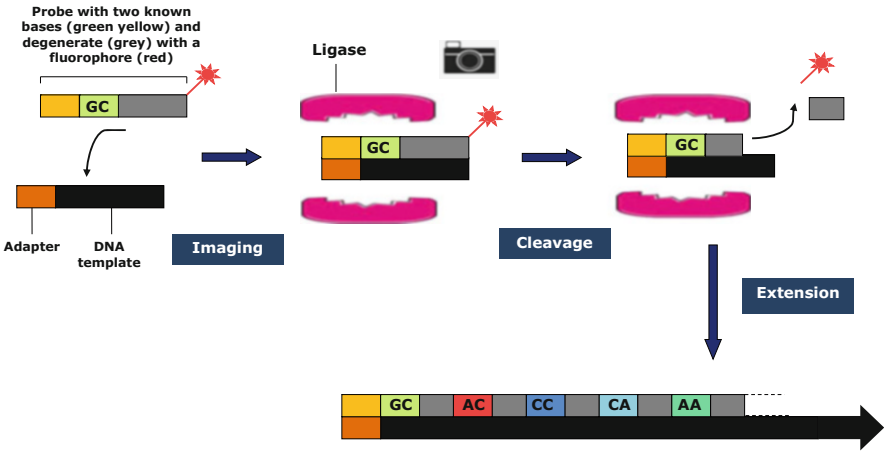


**Fig. 3.5** Principle of SOLiD sequencing**.** For comments, see text

### 3.3.2 BGI Complete Genomics Platforms (BGISEQ-500® and BGISEQ-50®)

BGISEQ sequencers are provided by the life sciences company "Complete Genomics" and use sequencing by ligation based on DNA nanoballs. In this technology, template preparation is performed utilizing a process called rolling circle amplification in which DNA undergoes repeated ligation, cleavage, and circularization (Goodwin et al. 2016) (Fig. 3.6a). After adapter ligation, template DNA is circularized and then cleaved downstream the adapter using endonucleases to bind other adapters in three additional cycles. Finally, the DNA is amplified to generate billions of circular structures that contain four adapters called nanoballs (Fig. 3.6b) to be deposited on sequencing flow cells (Goodwin et al. 2016; Drmanac et al. 2010). First, a complementary probe with single known base in addition to supplementary degenerate nucleotides and a fluorophore hybridize to the nanoball template via the sequences of the four ligated adapters. The complex is imaged and the probe is removed to enable hybridization of other new probes with another known base ($n$ + 1) in other rounds of sequencing cycles (Goodwin et al. 2016) (Fig. 3.6c). The company claims to have 99.999% accuracy in sequencing complete human genomes with only $600 (Dramanac et al. 2010; https://www.bgi.com/us/human-whole-genome-sequencing-from-600—accessed 27-05-2018). However, this technology is found to underrepresent AT-rich regions (Goodwin et al. 2016; Rieber et al. 2013). Using the BGISEQ-500 platform, some authors were able to show concordant results with Illumina HiSeq X10 in whole-genome sequencing of somatic and germline variants of pleural mesothelioma (Patch et al. 2018). Recently, a miniaturized and compacted desktop machine of BGISEQ-500 called BGISEQ-50 was released and designed for clinical sequencing laboratories with an output of 8 gigabases per run and a read length of 50 bp (https://www.genomeweb.com/

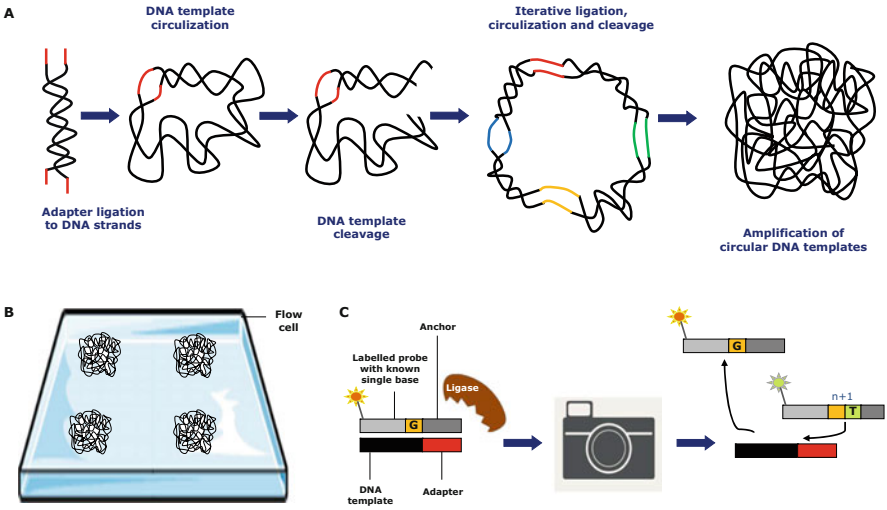


**Fig. 3.6** Principle of BGI Complete Genomics sequencing platforms: (**a**) template preparation, (**b**) immobilization of amplified DNA templates (known as nanoballs) on flow cells and hybridization, (**c**) hybridization of single-base probe to DNA template (nanoball) followed by imaging of the whole complex to identify the labeled base, removal of anchor-probe, and a new process begins with a new base ($n$ + 1 position). For additional comments, see text

sequencing/bgi-launches-new-desktop-sequencer-china-registers-larger-version-cfda#.WwsyczTRB0w—accessed 27-05-2018).

## 3.4 Real-Time Sequencing Platforms

The advent of single-molecule real-time sequencing technology used by Pacific BioSciences® and Oxford Nanopore® is based on considerably longer read generation of data without interruptions between read steps compared to the previously discussed technologies which produce short-read sequences (Goodwin et al. 2016; Bleidorn 2017).

### 3.4.1 Pacific BioSciences® (PacBio) Platforms

In PacBio technology, template preparation avoids clonal amplification by using direct sequencing of modified DNA (Rhoads and Au 2015). DNA templates are ligated to two hairpin barcoded adapters (Fig. 3.7a) followed by a removal of templates with inadequate size using a selection process (Goodwin et al. 2016). Templates and fluorescently labeled dNTPs are then deposited in picoliter wells called zero-mode waveguide cells containing each single DNA polymerase immobilized at the bottom that can bind the hairpin adapters (Rhoads and Au

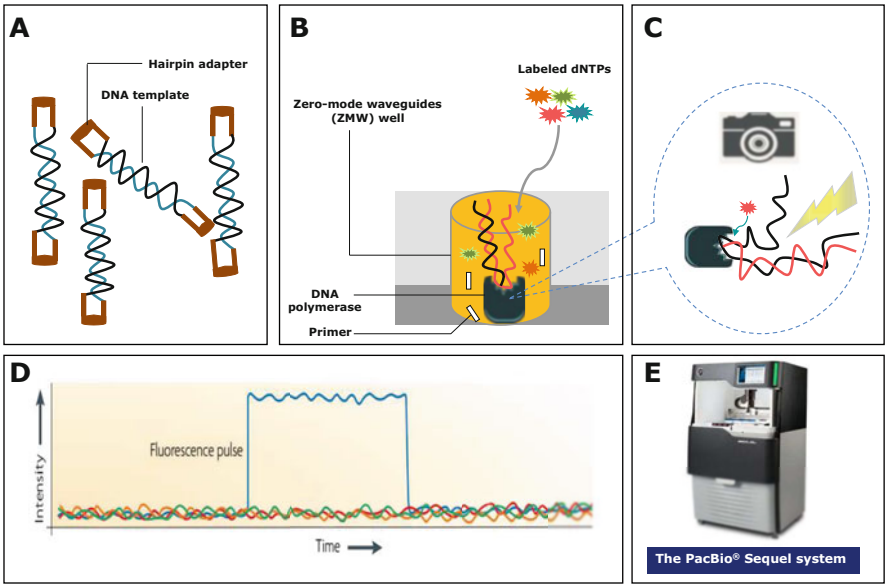


**Fig. 3.7** Principle of PacBio sequencing platform: (**a**) template preparation (ligation of hairpin adapters), (**b**, **c**) addition of prepared template into the zero-mode waveguide cells where real-time sequencing takes place, (**d**) example of a recorded fluorescence pulse (reprinted from Nat Rev Genet, 11, Metzker ML, Sequencing technologies-the next generation, 31–46, Copyright (2010), with permission from Springer Nature), (**e**) the recently launched PacBio Sequel system (reused with permission from Pacific Biosciences®). For comments, see text. *DNA* deoxyribonucleic acid, *dNTPs* deoxynucleotides, *ZMW* zero-mode waveguides

2015) (Fig. 3.7b, c). Resulting light pulses (Fig. 3.7d) corresponding to the colors emitted by the incorporated tagged nucleotides during amplification are detected and visualized using a camera and matched tags are cleaved off (Rhoads and Au 2015). With a great long read length estimated at ~20 Kb, PacBio RS II platform is the most commonly used for this purpose, and it seems to be the gold standard for de novo assembly of genome projects (Giordano et al. 2017; Goodwin et al. 2016; Gordon et al. 2016). However, this system is dominated by random indel errors, and their cost per gigabase is still high (Goodwin et al. 2016). To improve these drawbacks, PacBio has recently launched the PacBio Sequel system (Fig. 3.7e) that significantly ameliorated the sequencing throughput (~7× that of PacBio RS II) (Goodwin et al. 2016).

### 3.4.2 Oxford Nanopore Technologies® Platforms

Oxford Nanopore Technologies® (ONT) is a rising star in real-time sequencing using pocket-sized devices. Compared to the other platforms that detect secondary signals (pH changes, light emission, or color) revealing the composition of DNA, the

technology behind these long-read sequencers directly sequences DNA fragments during their passage through a biological protein nanopore fixed on a microwell (Goodwin et al. 2016; Clarke et al. 2009). Before sequencing, DNA is fragmented (8–10 kb) and ligated to two different adapters to form a leader-hairpin structure, a desired conformation that increases the interaction between the DNA and the α-hemolysin pore and facilitates its passage using a motor protein (Goodwin et al. 2016). Once the DNA is translocated through the pore, a characteristic disruption in the electric current is detected and enables a discrimination of nucleotides in question (Fig. 3.8a). In 2014, the company released its first attracting super-portable platform known as MinION (Fig. 3.8b) only with a price of $900, and able to sequence ~70 bp/s and adapted to personal laptops (Yang and Jiang 2017; Goodwin et al. 2016). Following its successful development, the company marketed two other multiple sequencing devices known as PromethION and GridION with up to 5–48 flow cells, respectively, which have increased dramatically its throughput (https://nanoporetech.com/how-it-works—accessed 04-06-2018). Very recently, the company has developed the VolTRAX, a small USB-powered manual device designed for automated library preparation without the need of a molecular biology laboratory and skilled sequencing teams (Fig. 3.8b). Moreover, another device called SmidgION for smallest sequencing purposes is being developed to be adapted for smartphone-based sequencing and will be launched soon. Importantly, Minervini et al. assessed *TP53* mutations in chronic lymphocytic leukemia by nanopore

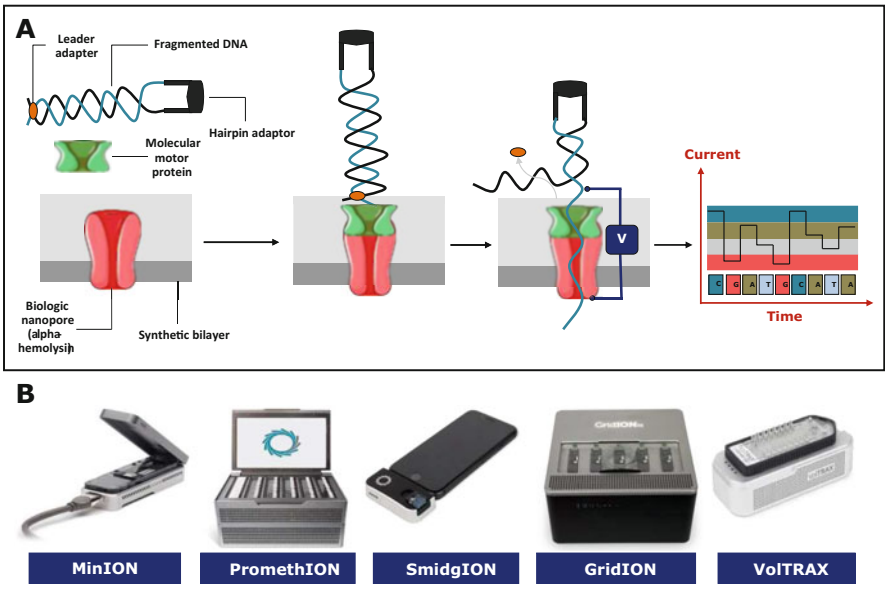


**Fig. 3.8** Principle of Oxford Nanopore sequencing: (**a**) summary of platforms sequencing principle, (**b**) pocket-sized devices developed recently by the company. For comments, see text

MinION and showed correlation, more sensitivity, and less expensiveness compared to Sanger sequencing (Minervini et al. 2016). However, despite these impressive advances, this nanopore sequencing is still suffering from high indel errors (other emerging sequencing technologies are listed in Table 3.1).

**Table 3.1** Other emerging next-generation sequencing technologies

| Company | NGS platform | Principle | Website |
|---|---|---|---|
| 10X Genomics[a] | Chromium and GemCode systems | Synthetic long-read and emulsion-based sequencing | https://www.10xgenomics.com/ |
| Direct Genomics | GenoCare | Single-molecule direct sequencing using TIRF[b] imaging for parallel detection of multiple fluorescently tagged single molecules | http://www.directgenomics.com/ |
| Bionano Genomics | Saphyr and Irys systems | High-resolution imaging of a linearized and labeled DNA in nanochannels | https://bionanogenomics.com/ |
| NanoString Technologies | Hyb and Seq | Library-free, amplification-free, single-molecule direct sequencing using cyclic DNA hybridization of fluorescent molecular barcodes | https://www.nanostring.com/ |
| GnuBio (Bio-Rad) | GnuBIO platform | Droplet microfluidics-based sequencing | http://gnubio.com/ |
| Genia (Roche) | In development | Single-molecule semiconductor-based DNA sequencing using nanopore technology | https://sequencing.roche.com/en/technology-research/technology/nanopore-sequencing.html |
| GenapSys | Genius | Electronic DNA sequencing | http://www.genapsys.com/ |
| Electron Optica | In development | Electron microscopy-based sequencing[c] | http://www.electronoptica.com/Electron_optica/HOME.html |
| IBM[d] | The DNA transistor (in development) | Nanopore-based sequencing | http://www-03.ibm.com/ibm/history/ibm100/us/en/icons/dnatransistor/ |
| NABsys | In development | Solid-state nanodetectors-based sequencing | http://nabsys.com/ |
| Electronic BioSciences | In development | Nanopore-based sequencing | http://electronicbio.com/ |

[a]Related publications can be found at: 10xgenomics.com/resources
[b]Total internal reflection fluorescence
[c]Details can be found at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4117835/ and http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0154707
[d]In collaboration with Roche

## 3.5 Conclusion

In conclusion, according to the current literature, the patents, and approvals for marketing, Illumina and Ion Torrent platforms seem to be the best mature sequencing devices to be used for clinical laboratory practice. Moreover, they are the most utilized for analyzing CRC genomics (see the next chapter for details; educative videos about NGS technologies can be found in Box 3.1). For further reading and useful websites, see Box 3.2.

**Box 3.1 Useful Links and Educative Videos About Next-Generation Sequencing Platforms and Technologies**

| Sequencing company | Website | Links for educative videos |
|---|---|---|
| Pyrosequencing (Roche®) | Discontinued | https://www.youtube.com/watch?v=KzdWZ5ryB1A |
| QIAGEN® PyroMark | https://www.qiagen.com/us/ | https://www.youtube.com/watch?v=bNKEhOGvcaI<br>https://www.youtube.com/channel/UCPXwu_KIrSKWMilWgiQuVaw<br>https://www.jove.com/video/50405/pyrosequencing-for-microbial-identification-and-characterization |
| Illumina® platforms | https://www.illumina.com/ | https://emea.illumina.com/science/technology/next-generation-sequencing/sequencing-technology.html<br>https://sapac.illumina.com/company/video-hub/view-all-videos.html<br>https://www.youtube.com/user/IlluminaInc |
| Thermo Fisher Ion Torrent® | https://www.thermofisher.com/ma/en/home/life-science/sequencing/next-generation-sequencing/ion-torrent-next-generation-sequencing-technology.html | https://www.youtube.com/watch?v=WYBzbxIfuKs |
| QIAGEN® GeneReader | https://www.qiagen.com/us/ | https://www.youtube.com/watch?v=HQhw5Ihp8IA |

(continued)

**Box 3.1** (continued)

| | | |
|---|---|---|
| Thermo Fisher SOLiD® | https://www.thermofisher.com/ma/en/home/life-science/sequencing/next-generation-sequencing/solid-next-generation-sequencing/solid-next-generation-sequencing-systems-reagents-accessories.html | https://www.thermofisher.com/ma/en/home/life-science/sequencing/next-generation-sequencing/solid-next-generation-sequencing/solid-next-generation-sequencing-systems-reagents-accessories.html<br>https://www.youtube.com/watch?v=YLT-DUeaLms |
| BGI Complete Genomics platforms | http://www.seq500.com/en/ | http://www.seq500.com/en/portal/videos.shtml |
| Pacific BioSciences® (PacBio) platforms | https://www.pacb.com/ | https://www.pacb.com/smrt-science/smrt-resources/video-gallery/ |
| Oxford Nanopore Technologies® platforms | https://nanoporetech.com/ | https://nanoporetech.com/resource-centre/videos<br>https://www.youtube.com/channel/UC5yMlYjHSgFfZ37LYq-dzig |

Additional videos about NGS can be found in JoVE (the *Journal of Visualized Experiments*): https://www.jove.com/

## Box 3.2 Useful Bioinformatic Tools, Websites, and Databases

| | |
|---|---|
| GeneCards®: The Human Gene Database | http://www.genecards.org/ |
| Online Mendelian Inheritance in Man® (OMIM) database | https://www.omim.org/ |
| The Cancer Genome Atlas Clinical Explorer[a] | http://genomeportal.stanford.edu/pan-tcga |
| The Catalogue Of Somatic Mutations In Cancer (COSMIC) | https://cancer.sanger.ac.uk/cosmic |
| Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer | https://cgap.nci.nih.gov/Chromosomes/Mitelman |
| Sequence Variant Nomenclature | http://varnomen.hgvs.org/ |
| ClinVar database[b] | https://www.ncbi.nlm.nih.gov/clinvar/ |
| Variant Annotation and Filter Tool[c] | http://varaft.eu/ |
| PharmGKB® | https://www.pharmgkb.org/ |
| GenomeWeb[d] | https://www.genomeweb.com |
| Cochrane Library | http://www.cochranelibrary.com/ |

(continued)

**Box 3.2** (continued)

| | |
|---|---|
| The U.S. National Library of Medicine clinical trials database | https://www.clinicaltrials.gov/ |
| The Human Gene Mutation Database (HGMD®) | http://www.hgmd.cf.ac.uk/ac/index.php |
| Guidelines for diagnostic next-generation sequencing | http://www.irdirc.org/guidelines-for-diagnostic-next-generation-sequencing/ |
| The International Society for Gastrointestinal Hereditary Tumours (InSiGHT) | https://www.insight-group.org/ |
| ASCO guidelines for molecular testing in colorectal cancer | https://www.asco.org/practice-guidelines/quality-guidelines/guidelines/gastrointestinal-cancer#/15831 |
| Educative videos about genomics | https://www.yourgenome.org/video |
| Colorectal Cancer Atlas | http://www.colonatlas.org/ |
| CoReCG[e] | http://lms.snu.edu.in/corecg/ |
| CBD: a biomarker database for colorectal cancer | http://sysbio.suda.edu.cn/CBD/ |
| Colon Cancer Alliance | https://www.ccalliance.org/ |
| The Human Pathology Atlas | http://www.proteinatlas.org/humanpathology/ |
| The Cancer Genome Atlas | https://cancergenome.nih.gov/ |
| IGSR: The International Genome Sample Resource | http://www.internationalgenome.org/ |

[a]A web and mobile interface for identifying clinical–genomic driver associations
[b]A database about genomic variations and their relationship to human health
[c]Details can be found in: https://academic.oup.com/nar/advance-article/doi/10.1093/nar/gky471/5025894
[d]An online news website focusing on genomics and emerging technologies
[e]A comprehensive database of genes associated with colon-rectal cancer

**Authors' Contribution** KE and FA wrote the chapter and MA supervised the writing process.

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# Next-Generation Sequencing for Colorectal Cancer Management

4

Khalid El Bairi and Said Afqir

**Abstract**

Recent sequencing reports provided huge amounts of actionable data about colorectal cancer (CRC) genomic landscape for various purposes including diagnosis and prediction of prognosis and response to treatments. Next-generation sequencing (NGS) tests enable refinement of the selection of CRC patients to benefit from recent targeted therapies. These advances constitute a rationale for the emerging targeted therapies that are changing the patients' outcomes. Notably, the advent of flexible next-generation sequencing (NGS) that allows multigene analysis is transforming our understanding of CRC. NGS workflow has successfully used to offer personalized medical care in CRC with a remarkable reduction of cost and time. However, this field is still facing many challenges regarding data analysis and development of bioinformatic tools as well as the clinical impact of the data obtained.

**Keywords**

Colorectal cancer · Next-generation sequencing · Diagnosis · Prognosis · Therapy response · Biomarkers

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## 4.1 Introduction

Previously adopted companion diagnostics based on traditional genotyping methods such as Sanger sequencing, PCR-based platforms, and DNA microarrays were used as a gold standard despite being expensive and time-consuming (Jørgensen 2015; Loree et al. 2017). Moreover, these techniques have a number of drawbacks regarding their sensitivity, scalability, high rate of amplification biases, low genome coverage, and the need of huge required amounts of high-quality DNA input which may not be achievable in formalin-fixed and paraffin-embedded (FFPE) tissue samples, a major source of nucleic acids in molecular pathology. To overcome these limitations, the arrival of "ready for action" NGS technology enables highly sensitive and massively parallel sequencing and simultaneous screening of various samples and multimarker panels (Morganti et al. 2019; Roy-Chowdhuri et al. 2019; Khotskaya et al. 2017). In addition, clinically actionable genetics has now successfully emerged into the management of CRC and it is moving this field toward a tailored personalized patients' care (Sandhu et al. 2019; Lin and Semrad 2018). Accurate understanding of human genetics related to CRC is critical to anticipate better clinical decision making. NGS allows a reliable generation of huge amounts of data associated with disease mechanisms including undescribed genetic variants with possible implication in CRC and multigene analyses that are not identified by the standard Sanger sequencing (Valle et al. 2019; Del Vecchio et al. 2017). Importantly, NGS provides high-quality and clinically actionable data for diagnosis, prognosis, therapy selection, and tracking tumor evolution and has seen a considerable evolution in the last few years which we discuss in this chapter based on recent published studies.

## 4.2 Next-Generation Sequencing Technologies for Colorectal Cancer Management

Accurate understanding of human genetics related to CRC is critical to anticipate better clinical decision making. NGS allows a reliable generation of huge amounts of data associated with disease mechanisms including undescribed genetic variants with possible implication in CRC and multigene analyses that are not identified by the standard Sanger sequencing (Del Vecchio et al. 2017). Remarkably, NGS provides high-quality and clinically actionable data for diagnosis, prognosis, therapy selection, and tracking tumor evolution and has seen a considerable evolution in the last few years.

### 4.2.1 Next-Generation Sequencing for Diagnosis

Management of CRC in the context of hereditary syndromes is critical to provide surveillance strategies and to predict disease risk. For diagnostic purposes, NGS can be performed to efficiently detect germline and somatic mutations using multigene

approaches. In addition, NGS-based genetic testing is powerful to confirm the routinely used immunohistochemistry to diagnose Lynch syndrome as well as to detect novel variants associated with CRC. In this scenario, for hereditary CRC, various NGS-based studies evidenced a variety of causative germline mutations allowing a reliable evaluation of genetic biomarkers. From a feasibility standpoint, Sapari et al. performed a low-throughput assay using Illumina MiSeq platform to assess their clinical and analytical performance for germline testing in Lynch syndrome samples (Sapari et al. 2014). This multigene panel includes 94 genes encompassing *MLH1*, *MSH2*, *MSH6*, and *PMS2* as well as 284 SNPs (Sapari et al. 2014).Targeted NGS detected all screened Lynch syndrome variants by Sanger sequencing but lacked high specificity. However, after polymorphism filtering, specificity and positive predictive value (PPV) were improved without compromising sensitivity (Sapari et al. 2014). In a previous report, Pritchard et al. developed a highly sensitive massively parallel and targeted NGS assay on the Illumina HiSeq 2000 instrument called ColoSeq that identifies mutations associated with Lynch syndrome and other polyposis syndromes (Pritchard et al. 2012). The investigators were able to detect 100% of pathogenic variants in seven validated genes including *MLH1*, *MSH2*, *MSH6*, *PMS2*, *EPCAM*, *APC*, and *MUTYH* (Pritchard et al. 2012). However, despite the fact that ColoSeq reduces the current stepwise testing for hereditary CRC syndromes, the power of this assay is limited by its long turnaround time (9 days) (Pritchard et al. 2012). In an attempt to determine the genetic alterations of hereditary cancers, LaDuca et al. analyzed the genome of 2029 patients with solid tumors including 557 hereditary CRC subjects using a panel of 14–22 genes on Illumina HiSeq 2000 (LaDuca et al. 2014). In this large-scale study, 46 subjects were positive for the well-established hereditary CRC genes and 5 subjects had mutations in the moderately penetrant checkpoint kinase 2 (*CHEK2*) tumor suppressor gene, suggesting that NGS-multigene panel is useful for the diagnosis of atypical phenotypes (LaDuca et al. 2014). Another recent large and multicenter study enrolled 419 CRC patients to assess whether tumor sequencing using NGS may avoid currently performed multiple sequential test approach for Lynch syndrome screening (Hampel et al. 2018). Screening using fluorescent multiplex PCR-based method, immunochemical staining, and $BRAF^{p.V600E}$ testing failed to detect some Lynch syndrome cases (Hampel et al. 2018). Importantly, tumor NGS alone improved sensitivity [100%; 95% CI, 93.8%–100%] compared to immunostaining + *BRAF* testing [89.7%; 95% CI, 78.8%–96.1%; $p = 0.04$] and multiplex PCR assay + *BRAF* testing [91.4%; 95% CI, 81.0%–97.1%; $p = 0.07$] as well as similar specificity (95.3%; 95% CI, 92.6%–97.2%) (Hampel et al. 2018). Furthermore, NGS provided actionable data regarding *KRAS*, *NRAS*, and *BRAF* mutations in 284 subjects which may avoid performing other tests for therapy purposes (Hampel et al. 2018). In this perspective, this study also found germline mutations in dihydropyrimidine dehydrogenase (*DPYD*) (Hampel et al. 2018), a known gene that confers toxicity to 5-FU and capecitabine (Deenen et al. 2011; Li et al. 2014). Moreover, NGS approaches are of significant value as they can differentiate Lynch syndrome from sporadic cancers which favors their wide use for universal screening of mismatch repair aberrations (Talseth-Palmer et al. 2016).

Additional findings from a report showed that mismatch repair protein deficiency and high microsatellite instability (MSI), which are key patterns of Lynch-associated CRC, can be assessed accurately using Illumina HiSeq2500-targeted sequencing (Nowak et al. 2017). Concordance with PCR results was seen for MSI analysis. In addition, NGS achieved 99% specificity and 100% sensitivity for MSI high in the training and validation sets (Nowak et al. 2017). In another recent report using the same NGS technology, a panel of 19 genes was used to identify patients with hereditary CRC syndromes from a cohort with a familial history or suspected CRC syndromes ($n = 91$) (Rohlin et al. 2017). The authors were able to identify 16 pathogenic or likely pathogenic variants and 30 variants of unknown significance. Notably, mutations in *BMPR1A* gene were identified in patients with pathogenic or likely pathogenic variants who had unexplained familial adenomatous polyposis (FAP) or atypical adenomatous polyposis (Rohlin et al. 2017). This is highly important for genetic counseling as it provides solutions for difficult situations related to significant genotype–phenotype diversity. The available NGS assays are changing our understanding of hereditary CRC syndromes; therefore, their rigorous qualitative validation for routine clinical practice must be performed (Tafe 2015).

In sporadic CRC, increased throughput and depth coverage allowed by NGS have (a) confirmed the previously reported CRC genetics by conventional Sanger sequencing, (b) revealed new candidate genes, (c) permitted high sensitive detection of low-frequency mutations and comprehensive profiling of heterogeneity, and (d) have the added advantage regarding the problem of low DNA template concentration from FFPE tissue samples compared with traditional Sanger sequencing. In an early feasibility study, whole exome pyrosequencing (Roche/454 FLX) of tumor and adjacent normal tissues of CRC patients with both MSS and MSI status detected more than 50,000 small nucleotide variants per tissue (Timmermann et al. 2010). After bioinformatic filtering, 359 and 45 significant mutations for both MSI and MSS tumors, respectively, were identified. As expected, this NGS approach revealed novel insights with regard to novel mutations and found that mutated *BMPR1A* associated with juvenile polyposis syndrome may also occur somatically in sporadic CRC (Timmermann et al. 2010). In addition, using Illumina Genome Analyser IIx, Han et al. analyzed CRC-associated somatic and copy number alterations by targeted sequencing with paired-end library enriched with exons of 183 genes (Han et al. 2013). Mutated *APC* (58%), *TP53* (57%), *KRAS* (40%), and genes associated with ERBB2 pathway (42%) were found to be the most frequent (Han et al. 2013). Consequently, targeted sequencing is a preferred alternative for clinical practice since whole-genome approaches provide other possible genetic alterations with poorly understood clinical importance. Later, analysis of DNA from 18 FFPE samples of CRC and lung cancer patients using a panel of 48 genes (TruSeq amplicon cancer panel) on Illumina MiSeq demonstrated that NGS identifies mutations in *EGFR* and *KRAS* genes that were considered as wild type when routine diagnostic tests were used (Chevrier et al. 2014). NGS may also identify less frequent somatic mutations in CRC with uncertain clinical significance such as *NOTCH1* (Notch homolog 1, translocation-associated) (0.2%), *AKT1* (AKT serine/threonine kinase 1) (0.9%), *STK11* (serine/threonine kinase 11) (0.8%), *ALK*

(anaplastic lymphoma kinase) (0.2%), and *FBXW7* (F-box/WD repeat-containing protein 7) (6%) but with possible therapeutic and predictive value (Malapelle et al. 2016). Importantly, one of the problems encountered in tissue-based genetic studies is the low quality of DNA derived from FFPE specimens which may limit the NGS excellence because of the high degree of fragmentation. Until recently, a PCR-based enrichment technique called AmpliSeq was designed for relatively short segments of genes and appears to be more compatible with altered DNA quality from tissue samples (Singh et al. 2013). Based on AmpliSeq (Ion Torrent PGM) that requires only 10 ng of DNA from CRC FFPE tissue samples, Zhang et al. sequenced 46 relevant genes in 44 FFPE specimens and found a high level of intertumor and intratumor heterogeneity (Zhang et al. 2014). However, AmpliSeq generated various recurrent false-positive calls in pharmacologically targetable genes including *PIK3CA*, and therefore, interpretation of these data is to be carried with caution (Zhang et al. 2014).

### 4.2.2 Next-Generation Sequencing for Prognosis and Therapy Response Prediction

Molecular testing of widely documented prognostic and predictive genes including *RAS* and *BRAF* is being practiced on the basis of various genetic assays including those using NGS technology. In this chapter, we will focus only on large and meticulously carried out studies, especially landmark clinical trials that investigated genetic biomarkers by NGS. In the pyrosequencing era, McLeod et al. evaluated 34 pharmacogenetic biomarkers that may predict adverse events or outcomes in germline DNA from 520 CRC patients enrolled in the North American Gastrointestinal Intergroup Trial N9741 which investigated three treatment arms [5-FU + irinotecan (arm A), FOLFOX (arm B), and IROX (irinotecan + oxaliplatin; arm C)] (McLeod et al. 2010). Genetic predictors including deleted glutathione S-transferase Mu 1 (*GSTM1*), UDP-glycosyltransferase 1 polypeptide A1 (*UGT1A1**28) polymorphism, and cytochrome P450 3A5 (*CYP3A5*) variant were found associated with grade 4 neutropenia (arm B; $p = 0.02$ and C; $p = 0.002$) and response rate (arm A, $p = 0.074$) (McLeod et al. 2010). In addition, CRC patients with *GSTP1* variant in the FOLFOX arm tend to terminate this regimen due to neurotoxicity ($p = 0.01$), thus supporting the use of these putative and robust predictive pharmacogenetic biomarkers in other clinical trials (McLeod et al. 2010). In the PICCOLO randomized and multicenter trial which has been designed to enroll patients with advanced CRC who have progressed under 5-FU treatment to receive irinotecan + cyclosporine (arm A) and irinotecan + panitumumab (arm B), molecular selection and patient stratification based on *KRAS* status were used, and significantly improved PFS (HR: 0.78; CI: 0.64–0.95, $p = 0.015$) and response rate (34%; $p < 0.0001$) were seen in subjects with wild-type *KRAS* (arm B) (Seymour et al. 2013). Similarly, data from a randomized phase III trial using massively parallel multigene panel demonstrated the feasibility of NGS to profile mutational landscape associated with response to panitumumab (Peeters et al. 2013). Mutated

*KRAS* and *TP53* were found to be the most prevalent (45% and 60%, respectively). Other potentially actionable mutated genes include *PIK3CA* (9%), *BRAF* (7%), *PTEN* (6%), *NRAS* (5%), *CTNNB1* (2%), *EGFR* (1%), and *AKT1* (<1%). Importantly, CRC subjects harboring wild-type *KRAS* treated with panitumumab had longer PFS as expected (HR: 0.39; CI: 0.28–0.56; 95%). Furthermore, wild-type *KRAS* CRC patients with wild-type *NRAS* and *BRAF* had better PFS (Peeters et al. 2013). Later, Ciardiello et al. assessed the mutational profile associated with outcomes in the CAPRI-GOIM trial treating metastatic CRC patients with first-line FOLFIRI + cetuximab (Ciardiello et al. 2014). Patients with wild-type *KRAS* and *NRAS* had better ORR [62% (95%; CI: 55.5–74.6%)] and median PFS (11.1 months, 95%; CI: 9.2–12.8) compared to the mutated status [ORR: 46.6%; median PFS: 8.9 months] (Ciardiello et al. 2014). This team also examined the efficacy of FOL FOX alone versus FOLFOX + cetuximab after progression in first-line FOLFIRI + cetuximab in a randomized phase II trial and evaluated tumor tissue samples by NGS in 117/153 CRC cases (Ciardiello et al. 2016). Remarkably, in the cohort with wild-type *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* ( FOLFOX + cetuximab), a significant prolongation of PFS was seen compared to the cohort treated with FOLFOX alone (HR: 0.56; CI: 0.33–0.94; 95%, $p = 0.025$) and therefore suggesting the potential use of anti-EGFR therapy when switching to another chemotherapy after progression in future RCTs as well as the importance of NGS in deep genetic profiling for patients' selection and stratification (Ciardiello et al. 2016). In a recent translational analysis of tumor samples from the PRIME study (NCT00364013), Udar et al. used the MiSeqDx® platform to evaluate the impact of *KRAS* and *NRAS* (exons 2, 3, and 4) on outcomes in a clinical trial that enrolled 528 metastatic CRC (Udar et al. 2018). This NGS-based panel allowed accurate patients' selection to benefit from anti-EGFR targeted therapy, and about 13% more patients were detected for inclusion as compared to Sanger sequencing (Udar et al. 2018). Strategies using multigene panels showed also superiority in detecting previously unreported prognostic variants in CRC (Domingo et al. 2018). This was evidenced in a clinical trial (QUASAR 2) that used multiple driver genes beyond *KRAS* and *BRAF* (Domingo et al. 2018). The Q UASAR 2 trial was an open-label randomized phase III that included 511 tumors from patients with stage II or III CRC (Domingo et al. 2018). Importantly, the authors identified two previously unreported variant association with CRC in *TP53* tumor suppressor gene (Domingo et al. 2018). Mutations in *TP53*, *KRAS*, *BRAF*, and *GNAS* were independent predictors of a significantly reduced relapse-free survival ($p < 0.035$) (Domingo et al. 2018). Moreover, this trial has also confirmed the negative association between mutated *KRAS* and *BRAF* with poor survival outcomes in MSI-negative tumors (Domingo et al. 2018). These important findings suggest that a modest-sized NGS panel can offer actionable information for future use in the management of CRC.

Data from real-world and validation studies also emerged to provide other insights into NGS as an alternative to the standard Sanger testing. To avoid adding cost, time, and matched normal tissue needed for MSI testing by PCR, Hempelmann et al. developed an NGS-based multimarker assay (MSIplus) which detects MSI in addition to *KRAS*, *NRAS*, and *BRAF* mutations simultaneously with a sensitivity of

97% and a superior detection limit (2%) of mutant allele fraction compared to previous methods (Hempelmann et al. 2015). However, these results must be interpreted with caution because of the low enrollment in this study. In a large CRC cohort ($n = 468$), a comparison between Illumina NGS and standard testing of predictive *KRAS* status demonstrated highly concordant results between the two methods [96.1% (95%; CI: 89%–99%)] (Kothari et al. 2014). NGS also showed superiority in detecting other mutations impacting the response to anti-EGFR therapy compared to the standard assays (including Sanger sequencing and other techniques) (Kothari et al. 2014). Moreover, another large comparative study ($n = 822$ including 168 FFPE CRC tissues) evaluated whether NGS has superior detection accuracy of mutated *KRAS*, *BRAF*, and *EGFR* than marketed FDA-cleared kits (Ma et al. 2017). NGS multimarker panels seem to have better accuracy for detection of mutations with clinical significance compared to FDA-approved assays which missed important mutated actionable genes, and therefore, it supports the notion that commercially available kits are inadequate in routine clinical laboratory testing (Ma et al. 2017). The search for flexible, accurate, and sensitive methods that are superior to standard assays has motivated some research teams to validate NGS, especially the small-sized Ion Torrent PGM sequencer that appears to be the best adapted option to the clinical laboratories environment. The Ion Torrent AmpliSeq Colon and Lung Cancer Panel is an attractive targeted NGS multimarker assay encompassing 1825 hotspots of 22 actionable genes associated with colon and lung cancers that has gained great consideration as a possible robust and cost-effective substitute (Malapelle et al. 2015). Potentially, D'Haene et al. retrospectively validate this panel using commercial reference standards followed by a retrospective analysis of 90 carcinoma cases (D'Haene et al. 2015). This is further supported by a prospective validation study that has shown a 100% concordance between this NGS assay and traditional Sanger sequencing (Belardinilli et al. 2015). Additionally, significant improvements were gained regarding costs and turnaround time (Belardinilli et al. 2015). Other findings from other studies are listed in Table 4.1. In addition to its place in diagnosis, prognostication, and prediction of treatment response, NGS technology has also illuminated the current model of colorectal carcinogenesis. Exome sequencing of primary tumors and metastases for example demonstrated that systemic spread in CRC can take place earlier during the tumorigenic process (Hu et al. 2019). Therefore, these significant advances may soon replace the traditional sequencing methods for CRC management particularly in the context of liquid biopsy (Bachet et al. 2018) (see Chap. 6 for a detailed discussion of this topic).

## 4.3 Tumor Sidedness, Next-Generation Sequencing, and Colorectal Cancer

Tumor sidedness (TSD) is another emerging prognostic and predictive factor in CRC (Zihui Yong et al. 2020; Chibaudel et al. 2020; Blakely et al. 2020; Lee et al. 2019). TSD has become a topic of great interest as it provides novel insights for patients'

**Table 4.1** Other key studies that investigated the role of NGS in prognosis and therapy response prediction in colorectal cancer

| Author/ year | Gene or gene panel | *N* | Tumor sample type | NGS platform | Findings |
|---|---|---|---|---|---|
| Ålgars et al. (2017) | Ion AmpliSeq™ panel[a] | 102 | FFPE | Ion Torrent PGM | • Copy number changes in *EGFR* predict therapy response to anti-EGFR monoclonal antibodies in wild-type RAS, *BRAF*, and *PIK3CA* metastatic CRC |
| Darwanto et al. (2017) | *KRAS* | 56 | FFPE | QIAGEN GeneReader | • GeneReader platform reached 100% concordance with standard *therascreen* RGQ PCR and Illumina MiSeq and is effective for somatic *KRAS* molecular profiling |
| Stadler et al. (2016) | MSK-IMPACT assay[b] | 224 | FFPE and blood samples | Illumina HiSeq 2500 | • Tumor genotyping using this NGS-based multigene approach provides high accuracy for MMR screening |
| Lupini et al. (2015) | 21-gene panel | 65 | FFPE | Ion Torrent PGM | • Mutated *FBXW7* and *SMAD4* were found frequent in resistant tumors to anti-EGFR therapy in addition to the already known mutated genes |
| Haley et al. (2015) | *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* | 314 | FFPE | Ion Torrent PGM | • NGS detected 17% of *KRAS* mutations outside the traditional codons 12 and 13 and 48% of *PIK3CA* mutations outside the codons 542, 545, and 1047<br>• Right-sided CRCs were at higher risk of resistance to targeted therapy |
| de Macedo et al. (2014) | *KRAS* and *BRAF* | 1 | FFPE | PyroMark Q24 ( QIAGEN) | • GGT insertions in *KRAS* gene revealed by pyrosequencing are very rare and clinically challenging because of the lack of data about their clinical effects |

*BRAF* v-raf murine sarcoma viral oncogene homolog B, CRCs: colorectal cancers, *FBXW7* F-box/WD repeat-containing protein 7, *FFPE* formalin-fixed paraffin-embedded, *KRAS* Kirsten rat sarcoma viral oncogene homolog, *NRAS* neuroblastoma RAS viral (v-ras) oncogene homolog, *N* enrollment, *NGS* next-generation sequencing, *PGM* personal genome machine, *PIK3CA* phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha, *SMAD4* mothers against decapentaplegic homolog 4

[a]KRAS, BRAF, NRAS, PIK3CA, EGFR, KIT, MET and PDGFRA

[b]Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) (details about this assay can be found in: Cheng et al. 2015)

stratification (Piawah and Venook 2019; Price et al. 2018). Left-sided tumors exhibit a markedly improved prognosis as compared to right-sided CRC in terms of survival (Papaxoinis et al. 2018; Tejpar et al. 2017; Sunakawa et al. 2017; von Einem et al. 2014). Moreover, anti-EGFR targeted therapy seems to be more effective in left-sided metastatic CRC, and antiangiogenics with chemotherapy are an optimal choice for right-sided tumors. This may be explained by the presence of a different regional distribution of prognostic and predictive mutations between right- and left-sided CRC. A pooled prevalence of genetic variants showed that mutated *KRAS* and *BRAF* are significantly more prevalent in right-sided tumors (46.3% and 16.3% respectively, $p < 0.0001$) (Bylsma et al. 2020). Additional relevant evidence from meta-analyses contributed significantly to further support TSD as an independent predictor of outcomes in CRC (Holch et al. 2017; Petrelli et al. 2017; Wu et al. 2020).

NGS technology was used in several studies to understand these patterns and identify the molecular profile of right-sided as compared to left-sided tumors. Based on NGS (Illumina MiSeq and NextSeq), Shimada et al. explored the genomic alterations in the EGFR pathway that may predict drug resistance in a cohort of 201 CRC patients using a panel of 415 genes (Shimada et al. 2017). 11% of patients with right-sided tumors had wild-type genotypes (Shimada et al. 2017). Those with mutated genotypes in the right-sided cohort had significantly reduced progression-free survival ($p = 0.004$) suggesting an association with resistance to the used targeted agents (Shimada et al. 2017). In another cohort, Loree et al. sequenced tumors of 1876 CRC patients based on the Ion Torrent PGM and compared their mutational profile based on TSD (Loree et al. 2018). Significant variations in mutation rates were noticed between left- and right-sided tumors. Remarkably, 70% of mutated *RAS* were observed in the cecal region followed by the hepatic flexure location (43%) in which mutated $BRAF^{V600}$ was more expressed (22%) (Loree et al. 2018). In the group with left-sided tumors, mutated *TP53*, *BRAF*, *CTNNB1*, and *PIK3CA* were more prevalent in the sigmoid and rectum (Loree et al. 2018). In terms of survival, right-sided tumors were significantly associated with short overall survival as compared to left-sided CRC after a period of 46.5 months of median follow-up (HR: 1.63, 95% CI: 1.38–1.89, $p < 0.0001$) (Loree et al. 2018). In the metastatic setting, tumors of 77 patients from phase II trials that investigated cetuximab as first line were sequenced using HTG EdgeSeq Oncology Biomarker Panel (2551 genes) in order to assess the molecular differences in CRC sidedness (Sunakawa et al. 2018). Importantly, patients with left-sided tumors in which *NOTCH1* was highly expressed had significantly improved progression-free survival as well as overall survival ($p = 0.01$ for both) (Sunakawa et al. 2018). These improved outcomes were not seen in right-sided CRC. Therefore, this single signature deserves to be further validated as a predictor of favorable response to cetuximab in left-sided CRC. A recent retrospective analysis of two phase II randomized trials ($n = 261$/NCT01161316 and NCT00885885) that studied the efficacy of anti-EGFR targeted agents (cetuximab or panitumumab) combined with chemotherapy in metastatic CRC confirmed the previous findings and demonstrated an improved efficacy in patients with wild-type *RAS* in left-sided

primary tumors (Benavides et al. 2019). Moreover, a higher risk for death and disease progression was noted in the right-sided group (Benavides et al. 2019). By using Illumina NextSeq, Salem et al. showed that right-sided CRC harbors additional mutated genes and epigenetic events involved in DNA repair and remodeling such as *KMT2D*, *ARID1A*, *MSH6*, *MLH1*, *MSH2*, *POLE*, *PTEN*, and *BRCA1* (Salem et al. 2019). Therefore, the application of NGS improves accuracy for detecting other variants that might explain some features in CRC beyond the well-known *RAS* and *BRAF* mutational status.

## 4.4 Tumor Mutational Burden and Next-Generation Sequencing in Colorectal Cancer

Tumor mutational burden (TMB) reflects highly mutated tumors having immunogenic neoantigens that may activate immune response. In a genomic profiling study of 100,000 human cancer genomes, TMB increased significantly with age (Chalmers et al. 2017). In addition, samples from patients with high microsatellite instability (MSI) status had high TMB (83%) (Chalmers et al. 2017). TMB and MSI together with PD1/PDL1 expression are predictors of response to immunotherapy (Luchini et al. 2019). Based on recent systematic reviews and meta-analyses, TMB is an emerging biomarker that demonstrated a potential to predict sensitivity to immune-checkpoint blockade in several cancer types (Kim et al. 2019; Wu et al. 2019). In CRC, this biomarker is reflective of survival outcomes and response to immune-checkpoint inhibitors (ICI) (Cohen et al. 2020). In an NGS-based study (FoundationOne; $n = 6004$) by Fabrizio et al., high TMB was observed in 164 of 5702 patients with microsatellite stable status, and therefore allowing the identification of a subgroup of patients to have a response to PD-1 inhibition (Fabrizio et al. 2018). Later, a case series of CRC patients ($n = 22$) treated with ICI showed that TMB was strongly associated with objective response ($p < 0.001$) and PFS ($p < 0.01$) (Schrock et al. 2019). The authors were able to provide an optimal predictive TMB cut-point between 37 and 41 mutations/Megabase using NGS (Schrock et al. 2019). More recently, primary tumor DNA from CRC patients ($n = 843$) who were enrolled in the CALGB/SWOG 80405 randomized phase III trial was sequenced using FoundationOne platform for TMB (Innocenti et al. 2019). High TMB predicted longer OS as compared to low TMB in the metastatic disease (HR: 0.73; 95% CI: 0.57–0.95; $p = 0.02$) (Innocenti et al. 2019). Importantly, the ESMO recommendations on microsatellite instability testing for immunotherapy in cancer stated that NGS for TMB and MSI status may become a decisive tool for patients' selection for immunotherapy indication (Luchini et al. 2019). However, mature data in CRC on this topic are not available yet for clinical practice.

## 4.5 In Clinical Practice

Currently, NGS technology is becoming mature, and it is used widely in practice for diagnosis, prognostication, and therapy response prediction supported by several landmark trials and international guidelines. For diagnostic purposes, the NCCN guidelines for screening (Version 3.2019) endorses germline multigene testing (preferred) and should include all polyposis and CRC genes. Moreover, the latest version of the NCCN guidelines for therapy (Version 2.2020) recommends the use of NGS panels for colon cancer to determine tumor gene status (*RAS*, *BRAF*, and *HER2* amplifications) as it has the advantage to pick up rare and actionable genetic variants as biomarkers for systemic therapy. Moreover, the US Food and Drug Administration (FDA) has recently released two guidelines that provide recommendations for designing, developing, and validating NGS-based tests (available at: https://www.fda.gov/media/99200/download / https://www.fda.gov/media/99208/download. Therefore, this will have an important impact in delivering genetic-based medicine in the future. However, several cons should be taken into consideration when applying NGS technology in clinical practice (depicted in Fig. 4.1).

## 4.6 Conclusion

The implementation of NGS technology in routine molecular diagnostic laboratories appears to have a superior place as a tool to predict prognosis and therapy response rather than for diagnostic purposes. Moreover, clinical application of NGS in oncology and particularly in CRC is valuable as biopsy specimens—which are the most used in practice—are limited by several issues including low quantity and quality of DNA inputs. To improve the accuracy, validation of these advances using other NGS platforms and Sanger sequencing is required before their implementation in the daily practice of oncologists. NGS produces huge amounts of data and novel variants but those with clinical relevance should be prioritized in further CRC research. Additional data from clinical trials assessing NGS technologies in CRC are summarized in Table 4.2. See Box 4.1 for recommended reading.

| PROS | CONS |
|---|---|
| -Can efficiently test more than one gene<br>-Superiority in multigene testing when more than one gene may explain cancer-associated syndromes<br>-Fast turnaround time for high sample volumes<br>-Lower cost than Sanger sequencing | -Higher chance of pathogenic variants identification for which clinical management is uncertain<br>-Provides huge amount of non-actionable findings<br>-Risk of results over-interpretation<br>-Lack of validated multigene panels and international guidelines |

**Fig. 4.1** Pros and cons of NGS technology in cancer management

**Table 4.2** Overview of some selected clinical trials investigating next-generation sequencing technology in colorectal cancer

| Study title[a] | Trial ID | Biospecimen | *N* | Sponsor |
|---|---|---|---|---|
| Technical Optimization of Detection of KRAS, BRAF and NRAS Mutations on Tumor DNA Circulating in Metastatic Colorectal Cancer (CircuLOR-1) | NCT02827565 | Circulating tumor DNA (ctDNA) and tumors embedded in paraffin | 30 | Institut de Cancérologie de Lorraine |
| Diagnosis of Lynch Syndrome Based on Next-generation Sequencing in Colorectal Cancer | NCT03047226 | Whole blood, frozen tissue, paraffin tissue, formalin-fixed tissue, and RNA-later preserved tissue | 300 | Second Affiliated Hospital, School of Medicine, Zhejiang University |
| Targeted Next-generation Sequencing Panel for Identification of Germline Mutations in Early Onset Cancers With Sporadic or Hereditary Presentation (PANEL) | NCT02664389 | Not specified | 600 | University Hospital, Rouen |
| Systemic Screening for Hereditary Colorectal Cancer in China | NCT03365986 | Whole blood | 500 | Sun Yat-sen University |
| Diagnosis of Lynch Syndrome Based on Next-generation Sequencing in Patients Meeting Chinese Lynch Syndrome Criteria | NCT03046849 | Whole blood | 200 | Second Affiliated Hospital, School of Medicine, Zhejiang University |
| Investigation of the Value of ctDNA in Diagnosis, Treatment, and Surveillance of Surgically Resectable Colorectal Cancer | NCT03038217 | ctDNA | 300 | Peking Union Medical College Hospital |

(continued)

**Table 4.2** (continued)

| Study title[a] | Trial ID | Biospecimen | *N* | Sponsor |
|---|---|---|---|---|
| A Randomized, 2×2 Factorial Design Biomarker Prevention Trial of Low-dose Aspirin and Metformin in Stage I-III Colorectal Cancer Patients ( ASAMET) | NCT03047837 | Normal colonic tissue | 160 | Ente Ospedaliero Ospedali Galliera |
| Ohio Colorectal Cancer Prevention Initiative (OCCPI) | NCT01850654 | Tumor, blood, and saliva samples | 3470 | Ohio State University Comprehensive Cancer Center |
| Ontario-wide Cancer TArgeted Nucleic Acid Evaluation ( OCTANE) | NCT02906943 | Blood samples and additional archival tumor specimens | 10,000 | University Health Network, Toronto in collaboration with Princess Margaret Hospital, Canada |
| Anti-EGFR Therapy Rechallenge in Combination With Chemotherapy in Patients With Advanced Colorectal Cancer (A-REPEAT) | NCT03311750 | ctDNA | 33 | Hellenic Cooperative Oncology Group in |
| Comprehensive Gene Sequencing in Guiding Treatment Recommendations Patients With Metastatic or Recurrent Solid Tumors | NCT01987726 | Tissue and blood samples | 150 | Ohio State University Comprehensive Cancer Center in collaboration with Foundation Medicine |
| CureOne Registry: Advanced Malignancy or Myelodysplasia, Tested by Standard Sequencing and Treated by Physician Choice (N1) | NCT02900248 | Tumor tissue | 100,000 | CureOne |

(continued)

**Table 4.2** (continued)

| Study title[a] | Trial ID | Biospecimen | *N* | Sponsor |
|---|---|---|---|---|
| GENESIS: Genetic Biopsy for Prediction of Surveillance Intervals After Endoscopic Resection of Colonic Polyps ( GENESIS) | NCT02595645 | Polyp tissue and ctDNA samples | 101 | University of Ulm in collaboration with Technische Universität München, Medical University of Graz, Specialized Medical Office for Gastroenterology Dornstadt, and QI AGEN Gaithersburg, Inc. |
| PROSPECT-C: A Study of Biomarkers of Response or Resistance to Anti-EGFR Therapies in Metastatic Colorectal Cancer ( PROSPECT-C) | NCT02994888 | Fresh frozen tissue, formalin-fixed paraffin-embedded (FFPE) tissue, and blood samples | 47 | Royal Marsden NHS Foundation Trust |
| Identification of Predictive Biomarker of Regorafenib in Refractory Colorectal Cancer | NCT01996969 | Surgical FFPE or fresh-frozen biopsy | 117 | Seoul National University Hospital in collaboration with Bayer |
| Comprehensive Genomic Profiling of Colorectal Cancer Patients With Isolated Liver Metastases to Understand Response & Resistance to Cancer Therapy ( COMPARISON) | NCT03364621 | Fresh tumor tissue and whole blood | 20 | University Health Network, Toronto, in collaboration with Terry Fox Research Institute and British Columbia Cancer Agency |
| Feasibility Study of Genomic Profiling Methods and Timing in Tumor Samples | NCT01703585 | Blood and tumor tissue samples | 45 | University Health Network, Toronto, in collaboration with Ontario Institute for Cancer Research and Princess Margaret Hospital, Canada |

*BRAF* v-raf murine sarcoma viral oncogene homolog B, *ctDNA* circulating tumor DNA, *EGFR* epidermal growth factor receptor, *FFPE* formalin-fixed paraffin-embedded, *KRAS* Kirsten rat sarcoma viral oncogene homolog, *NRAS* neuroblastoma RAS viral (v-ras) oncogene homolog, *RNA* ribonucleic acid

[a]Titles of the clinical trials were copied as shown by the database. Data in this table were taken from ClinicalTrials.gov as of 19/12/2017, a combination of keywords including colorectal cancer and next-generation sequencing were used. Trials with few data about the sequencing methods were excluded

### Box 4.1 Recommended Articles from Highly Accessed *Medline*-Indexed Journals

| | |
|---|---|
| Thavaneswaran S, et al. *Therapeutic implications of germline genetic findings in cancer*. Nat Rev Clin Oncol. 2019;https://doi.org/10.1038/s41571-019-0179-3. | doi:https://doi.org/10.1038/s41571-019-0179-3 |
| Ho SS, et al. *Structural variation in the sequencing era*. Nat Rev Genet. 2019;https://doi.org/10.1038/s41576-019-0180-9. | doi:https://doi.org/10.1038/s41576-019-0180-9 |
| Yamamoto H, Imai K. *An updated review of microsatellite instability in the era of next-generation sequencing and precision medicine*. Semin Oncol. 2019;46(3):261–270. | doi:https://doi.org/10.1053/j.seminoncol.2019.08.003 |
| Reilly NM, et al. *Exploiting DNA repair defects in colorectal cancer*. Mol Oncol. 2019;13(4):681–700. | doi:https://doi.org/10.1002/1878-0261.12467 |
| Berger MF, Mardis ER. *The emerging clinical relevance of genomics in cancer medicine*. Nat Rev Clin Oncol. 2018;15(6):353–365. | doi:https://doi.org/10.1038/s41571-018-0002-6 |
| Hardwick SA, et al. *Reference standards for next-generation sequencing*. Nat Rev Genet. 2017;18 (8):473–484. | doi:https://doi.org/10.1038/nrg.2017.44 |
| Samuels DC1, et al. *Finding the lost treasures in exome sequencing data*. Trends Genet. 2013;29(10):593–9. | doi:https://doi.org/10.1016/j.tig.2013.07.006 |
| Kilpinen H, Barrett JC. *How next-generation sequencing is transforming complex disease genetics*. Trends Genet. 2013;29(1):23–30. | doi:https://doi.org/10.1016/j.tig.2012.10.001 |
| Reuter JA, et al. *High-throughput sequencing technologies*. Mol Cell.2015;58(4):586–97. | doi:https://doi.org/10.1016/j.molcel.2015.05.004 |
| Song Y, et al. *Point-of-care technologies for molecular diagnostics using a drop of blood*. Trends Biotechnol. 2014;32(3):132–9. | doi:https://doi.org/10.1016/j.tibtech.2014.01.003 |
| Koboldt DC, et al. *The next-generation sequencing revolution and its impact on genomics*. Cell. 2013;155 (1):27–38. | doi:https://doi.org/10.1016/j.cell.2013.09.006 |
| Kraus VB. *Biomarkers as drug development tools: discovery, validation, qualification and use*. Nat Rev Rheumatol. 2018;14(6):354–362. | doi:https://doi.org/10.1038/s41584-018-0005-9 |
| Oliver GR, et al. *Bioinformatics for clinical next generation sequencing*. Clin Chem. 2015;61(1):124–35. | doi:https://doi.org/10.1373/clinchem.2014.224360 |

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# 5 Gut Microbiota, Next-Generation Sequencing, Immune-Checkpoint Inhibitors, and Colorectal Cancer: How Hot Is the Link?

Khalid El Bairi, Mariam Amrani, and Adil Maleb

**Abstract**

The promising high-throughput metagenomics sequencing including both taxonomic 16S ribosomal RNA and shotgun sequencing has greatly facilitated the study of gut microbiota and its association with disease. Intestinal microbiota influences host immunity and may profoundly interact with mucosal environment and induce its perturbation by various mechanisms involving genetic and metabolic by-products. Current evidence suggests a primary and fundamental role of microbiota dysbiosis in driving the progress of the adenoma-carcinoma sequence and metastasis of colorectal cancer (CRC). Interestingly, microbiome dysbiosis-based signatures are promising biomarkers to predict prognosis, therapy response to cancer immunotherapy, drug adverse events, as well as survival outcomes. The focus of this chapter is to critically review the role of gut microbiota in colorectal carcinogenesis and its potential in biomarker development and progress of the rapidly evolving oncoimmunology. We also discuss the perspectives of using microbiota in the clinical decision-making workflow.

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**Keywords**

Colorectal cancer · Gut microbiota · Metagenomics · Biomarkers · Cancer immunotherapy

## 5.1 Introduction

The entire immune system is deeply influenced by gut microbiota that modulates distinct signaling pathways related to homeostatic immune response. Notably, gut microbiota plays a remarkable role in anticancer immunosurveillance and general health (Routy et al. 2018a). However, the number of cancers associated with human microbiome dysbiosis is rising, particularly with the advent of sophisticated techniques such as metagenomic sequencing (Jiang et al. 2019; Sze and Schloss 2018; Mao et al. 2018; Mani 2017; Yang et al. 2017a, b; Chen et al. 2017; Shah et al. 2017). Diverse pathogenic organisms and passenger commensals may be implicated in gut flora dysbiosis, in addition to other factors such as antibiotics, aging, alcohol consumption, and diet composition as well as environmental factors which are also well-known risk factors for neoplastic transformation (Zitvogel et al. 2015). The list of bacteria associated with CRC is far to be completed, but currently, there is a trend to focus on particular organisms such as the famous anaerobic oncobacterium *Fusobacterium nucleatum* (Mima et al. 2015; Brennan and Garrett 2019; Ramos and Hemann 2017; Han 2015). Moreover, gut microbiota influences adverse events and response to chemotherapy and, therefore, can be targeted to improve the efficacy of pharmacological effects (Alexander et al. 2017). Interestingly, intestinal microbiome baseline characteristics correlates with clinical response to the cutting-edge cancer immunotherapy including monoclonal antibodies targeting PD-1/PD-L1 axis (programmed cell death protein 1/programmed cell death 1 ligand 1) and cytotoxic T-lymphocyte protein 4 (CTLA-4) (Derosa et al. 2018a; Kroemer and Zitvogel 2018; Pitt et al. 2016). Therefore, it seems that gut microbiota is actionable and may be manipulated to improve outcomes using interventional procedures such as fecal microbiota transplantation (Chen et al. 2018; Wang et al. 2018). Recent experimental and epidemiological studies provided evidence to illuminate the cardinal association between dysbiosis of gut microbiota and CRC which is the core of this chapter. We also extend our discussion to a special focus on molecular mechanisms of this association and their potential in cancer biomarker discovery in order to select the right patients to benefit from the emerging immune-checkpoint inhibitors (ICI).

## 5.2 Gut Microbiota Dysbiosis: A Focus on General Mechanisms

Gut microbiota dysbiosis is a rising driver that may contribute in promoting colorectal carcinogenesis (Burns et al. 2018; Zou et al. 2017; Louis et al. 2014; Brennan and Garrett 2016) as well as other diseases (Sekirov et al. 2010) (Fig. 5.1). There is

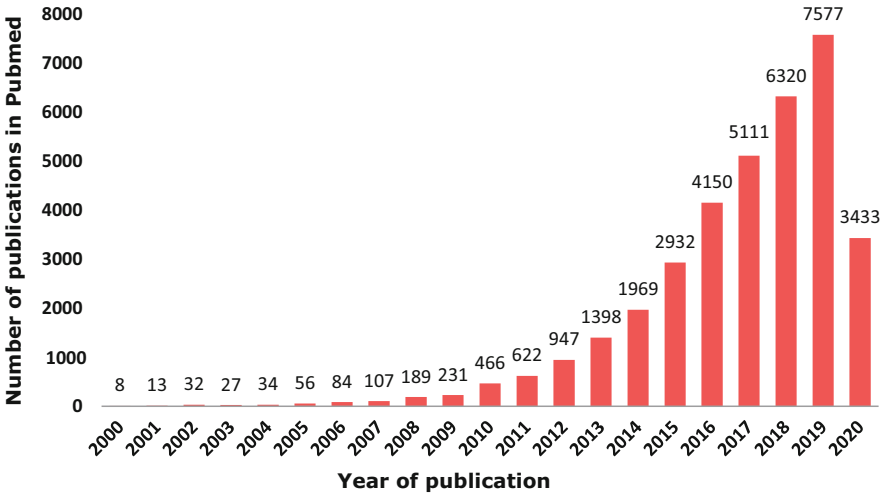



**Fig. 5.1** Evolution of research on gut microbiota in disease according to PubMed (visualized by the number of published articles accessed 01/05/2020)

growing evidence supporting the link between microbiome metabolism, pro-inflammatory microenvironment, and growth and metastasis of CRC through a mechanism involving carcinogenic bacteria (Mima et al. 2015; Louis et al. 2014; Dejea et al. 2018). Human gut microbiota is very dense and diverse and is dominated by anaerobic microbial flora in the intestinal lumen or adhered to the mucosa (Louis et al. 2014). In addition to their dynamic and symbiotic role in immunity and digestion, they are also involved in gut dysfunction including inflammatory bowel disease and CRC (Keku et al. 2015). Several factors such as diet, lifestyle, antibiotics intake, and infections can degenerate this homeostatic relationship and lead to the overgrowth of opportunistic microbes (Schwabe and Jobin 2013; Keku et al. 2015). Mechanistically (Fig. 5.2), during infective invasion, an increase in epithelial permeability is observed allowing the translocation of bacteria mediating a host-immune response via microorganism-associated molecular patterns (MAMPS) (Schwabe and Jobin 2013). MAMPS are immunologically recognized by local macrophages, epithelial cells, and other immune cells via cell surface Toll-like receptors (TLRs); they enable a pro-inflammatory cytokine secretion of tumor necrosis factor (TNF-α), interleukin 1 (IL-1), and interleukin 6 (IL-6) that orchestrate the host sustained proliferative response through NF-κB pathway, a hallmark of cancer (Schwabe and Jobin 2013; Louis et al. 2014). Furthermore, recognition of MAMPS by dendritic cells leads to the activation of $T_H17$ (T helper 17) which in turn supports the inflammatory microenvironment (Louis et al. 2014). DNA damage caused by bacterial genotoxins such as colibactin produced by *Escherichia coli* is also believed to drive colorectal tumorigenesis through the possible induction of genomic instability (Louis et al. 2014; Brennan and Garrett 2016). In addition, bacterial metabolism is a major source of carcinogenic compounds that can alter

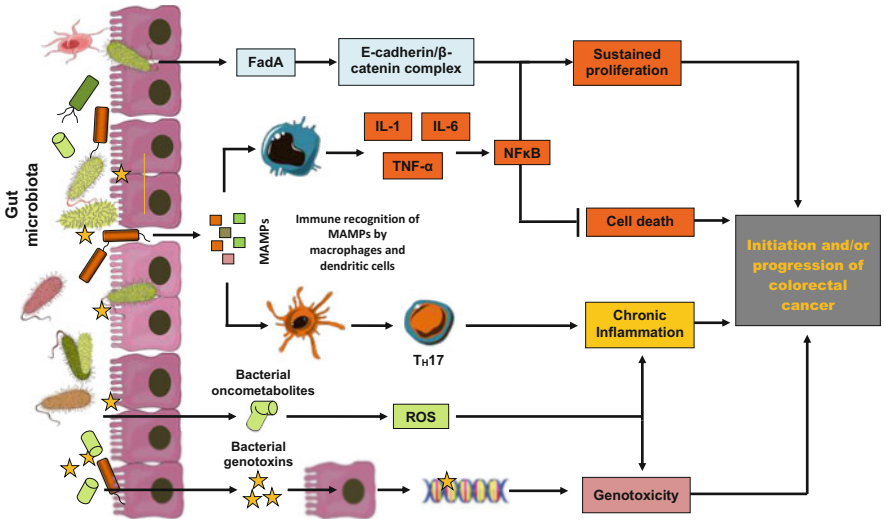



**Fig. 5.2** Mechanistic pathways involved in the cross-talk between gut microbiota and colorectal carcinogenesis. For comments, see text. *FadA* Fusobacterium adhesin A, *IL-1* interleukin 1, *IL-6* interleukin 6, *MAMPs* microbe-associated molecular patterns, *NFκB* nuclear factor-kappa B, *ROS* reactive oxygen species, *Th17* T helper 17, *TNF-α* tumor necrosis factor alpha

the colon epithelium by inducing inflammation or direct damaging of DNA (Louis et al. 2014). High consumption of proteins and fatty acids and ethanol intake are well-established factors involved in the production of toxic products by gut microbiota including secondary organic bile acids, *N*-nitroso compounds, hydrogen sulfides, acetaldehyde, and other carcinogens, all known for their genotoxicity, strong generation of reactive oxygen species (ROS), and inflammation (Schwabe and Jobin 2013; Louis et al. 2014). Another established mechanism mediating the carcinogenic effects of gut microbiota with CRC is the unique FadA adhesin of *Fusobacterium nucleatum* which after binding to E-cadherin/β-catenin signaling pathway activates oncogenic cell growth (Rubinstein et al. 2013) (Box 5.1). Moreover, inhibition of immune cell activity by intratumor fusobacterial protein Fap2 was also recently noted (Gur et al. 2015) which was also inversely associated with $T_{CD3+}$ cell density in CRC tissue (Mima et al. 2015). According to these study results, Fap2 protein binds to immunoreceptor TIGIT (T-cell immunoreceptor with Ig and ITIM domains) present on NK cells and, therefore, inhibits their antitumor activity (Gur et al. 2015). This team has further demonstrated that Fap2 also inhibits the functions of tumor-infiltrating lymphocytes (TILs) expressing TIGIT (Gur et al. 2015). Fap2 protein recognizes and interacts with host overexpressed Gal-GalNAc (D-galactose-β (1–3)-*N*-acetyl-D-galactosamine) on CRC cells and enables enrichment of local *Fusobacterium nucleatum* which might exacerbate its cancer-promoting activity (Abed et al. 2016). Gal-GalNAc is a lectin that was proposed as potential biomarker for CRC in the 1990s (Yang and Shamsuddin 1996) and seems to be targetable for CRC drug discovery (Abed et al. 2016). Interestingly, this previous report noted that

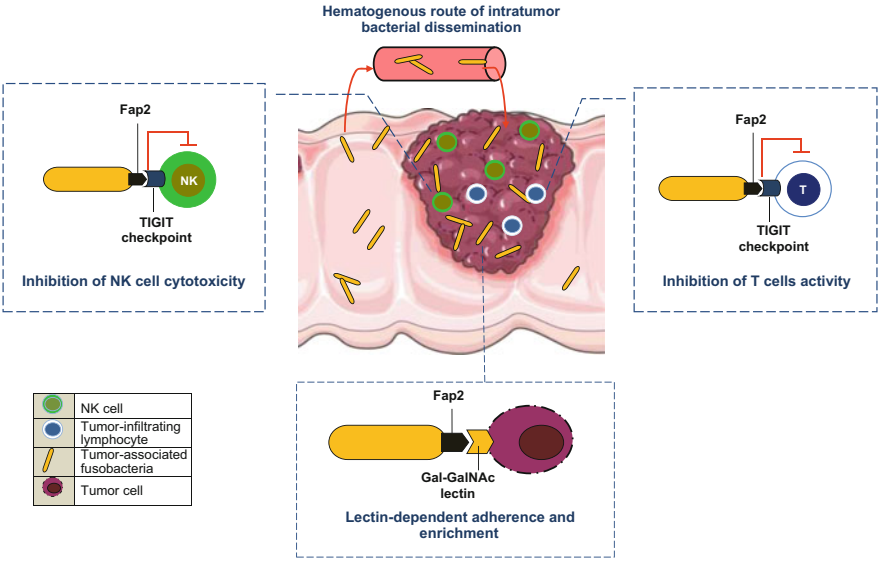



**Fig. 5.3** Mechanisms of fusobacterial enrichment, dissemination, and immune cell inhibition in colorectal tumors. For comments, see text. *Fap2* Fusobacterium nucleatum outer membrane protein 2, *Gal-GalNAc* D-galactose-β(1–3)-*N*-acetyl-D-galactosamine, *TIGIT* T-cell immunoreceptor with Ig and ITIM domains

*Fusobacterium nucleatum* reaches CRC tissues using hematogenous route in a Fap2-dependent manner (Abed et al. 2016) (Fig. 5.3). Promisingly, immunogenic peptides of Fap2 were recently used in a proof-of-concept study to develop blood-based diagnostic biomarkers for CRC (Guevarra et al. 2018). Along these lines and in light of this growing body of evidence, all of these factors as well as the host-specific genetics may promote development of CRC or malignant transformation of adenoma into carcinoma (for more details, see the bacterial driver–passenger model by Tjalsma et al. 2012).

## 5.3 The Impact of Next-Generation Sequencing in Understanding the Association of Gut Microbiota with Colorectal Carcinogenesis

With the remarkable advances in NGS technology and metagenome association studies (MGWAS), revelation of pathophysiological interactions linking microbiome complexity and CRC is now more understandable and could represent potential applications to understand disease mechanisms and therapeutic modulation (Allen and Sears 2019; Wang and Jia 2016; Ji and Nielsen 2015). During the last 5 years, various proof-of-concept and associative studies (Table 5.1) have attempted to dissect the role of gut microbiota in driving CRC using advanced sequencing technologies such as Illumina MiSeq which is adapted to small genomes. In most of

**Table 5.1** Key studies using NGS technology for studying microbiota in CRC from the last 5 years

| Author/Year | Sequencing technology | Sampling | Findings |
|---|---|---|---|
| Russo et al. (2018) | Illumina MiSeq | Saliva, feces, and tumor specimens | • 16S rRNA gene sequencing revealed the presence of three principal bacterial phyla including 39.18% of Firmicutes, 30.36% of *Bacteroidetes,* and 10.65% *Proteobacteria* (80% of reads)<br>• Fecal samples from CRC patients seemed to be enriched with *Bacteroidetes* |
| Wong et al. (2017) | Illumina MiSeq | Feces | • Gavage of fecal samples from CRC cases to germ-free mice was found to increase the number of polyps, colonocyte proliferation and dysplasia, inflammatory biomarkers, as well as proportions of $T_H1/T_H17$ lymphocytes in colon as compared with mice fed with samples from healthy cases |
| Hibberd et al. (2017) NCT03072641 | Illumina MiSeq | Feces, tumor, and normal mucosa samples | • Tumor samples and adjacent mucosa of CRC patients carried a distinctive microbiota signature (*Fusobacterium*, *Selenomonas,* and *Peptostreptococcus*) which was altered after prebiotic treatment with *Bifidobacterium lactis* and *Lactobacillus acidophilus* |
| Bullman et al. (2017) | Illumina MiSeq and Illumina HiSeq2500 | Tumor samples (primary and metastases) | • Viable CRC-associated *Fusobacterium* and other gut microbiota (*Bacteroides*, *Selenomonas*, and *Prevotella* species) are maintained in distant metastatic tumors |
| Yu et al. (2017a) | Illumina HiSeq 2000 | Feces | • This study confirmed the association between CRC and *Fusobacterium nucleatum* and *Peptostreptococcus stomatis* as well as novel associations with various bacterial species including *Parvimonas micra* and *Solobacterium moorei*<br>• This metagenomic study further identified 20 microbial genes as noninvasive biomarkers that may distinguish CRC-associated and control microbiomes |
| Purcell et al. (2017) | Illumina MiSeq and Illumina HiSeq 2500 | Tumor samples | • 16S rRNA gene sequencing revealed an enrichment of *Fusobacteria* and *Bacteroidetes*, and low abundance of *Firmicutes* and *Proteobacteria* in consensus molecular subtypes 1 (CMS1). Enrichment of *Selenomonas* and *Prevotella* species was seen in CMS2 |

| | | | |
|---|---|---|---|
| Drewes et al. (2017) | Illumina MiSeq | Mucosal samples from healthy cases and tumor/normal samples from CRC patients | • CRC samples were found to be enriched by invasive biofilms and tumorigenic bacteria (*Fusobacterium nucleatum*, *Bacteroides fragilis*, *Parvimonas micra*, and *Peptostreptococcus stomatis*) |
| Flemer et al. (2017a) | Illumina MiSeq | Fecal and mucosal samples | • Gut microbiota profiles in CRC vary from those in healthy individuals as well as between tumor localization |
| Flemer et al. (2017b) | Illumina MiSeq | CRC samples, oral swabs, colonic mucosa, and feces | • Oral taxa including *Prevotella spp.* and *Streptococcus* were differentially abundant in CRC compared with healthy subjects<br>• Highly abundant *Lachnospiraceae* bacteria were found negatively associated with the presence of oral-like bacteria in colon tissue samples suggesting their possible protective roles against CRC occurrence |
| Peters et al. (2016) | Illumina MiSeq | Feces | • Diversity of gut microbiota was found to be decreased in colonoscopy-screened cases with conventional colorectal adenoma-associated changes compared to controls<br>• Cases with advanced conventional adenomas had more reduced community diversity |
| Sinha et al. (2016) | Roche/454 GS-FLX Titanium (pyrosequencing) | Feces | • Very strong microbe–metabolite correlations with predominance of *Enterobacteriaceae* and *Actinobacteria* were seen in feces of CRC patients |
| Thomas et al. (2016) | Ion Torrent PGM | Mucosal samples from healthy cases and tumor samples from rectal cancer patients | • High-throughput NGS of 16S rRNA amplicon showed that rectal tumors had a significantly increased species' diversity and richness based on OTU analysis |
| Zackular et al. (2016) | Illumina MiSeq | Feces | • In this murine study based on antibiotic perturbations, treating gut microbiota in the midst of colorectal carcinogenesis could reduce the occurrence of other tumors |
| Feng et al. (2015) | Illumina (not specified) | Feces | • A metagenomic analysis showed that high intake of red meat seems to be associated with bacteria dysbiosis that might contribute in CRC development |
| Goedert et al. (2015) | Illumina MiSeq | Feces | • Composition of fecal microbiota differs significantly between patients with adenoma and those with normal colonoscopy |

(continued)

**Table 5.1** (continued)

| Author/Year | Sequencing technology | Sampling | Findings |
|---|---|---|---|
| Gao et al. (2015) | Roche/454 GS-FLX (pyrosequencing) | Tumor tissue and adjacent non-tumor normal tissue samples | • A significant difference between microbial structures of CRC cases and healthy individuals was seen<br>• Firmicutes and *Fusobacteria* were overrepresented in CRC individuals compared to *Proteobacteria*<br>• *Lactococcus* and *Fusobacterium* showed a relatively higher abundance in CRC tissues compared to adjacent normal tissues |
| Mira-Pascual et al. (2015) | Roche/454 GS-FLX Titanium (pyrosequencing) | Mucosal and fecal samples | • A higher presence of *Fusobacterium nucleatum* was seen in CRC tissue and feces samples compared to controls |
| Nakatsu et al. (2015) | Roche/454 GS-FLX Titanium (pyrosequencing) | • Mucosal samples from adenoma cases and their adjacent tissue<br>• Mucosal samples from carcinoma and their adjacent tissue and mucosal samples from healthy cases | • Microbial communities cataloging at different stages of colorectal carcinogenesis revealed that oral microbiome metacommunity is associated with this cancer. Also, an early and co-exclusive microbiome dysbiosis is seen in adenoma and CRC, respectively |
| Zeller et al. (2014) | Illumina MiSeq and Illumina HiSeq 2000/2500 | Feces | • Accuracy of detection of CRC based on metagenomics was similar to the standard fecal occult blood test (FOBT) |
| Zackular et al. (2014) | Illumina MiSeq | Feces | • Gut microbiome in combination with known risk factors of CRC significantly improved the ability to differentiate between healthy individuals, adenoma, and carcinoma cases |
| Tahara et al. (2014) | Illumina HiSeq 2000 | Tumor tissues, adjacent normal mucosa, and colonic mucosa from cancer-free individuals | • Exome sequencing showed that CRC cases with a high abundance of *Fusobacterium* had the highest number of somatic mutations |
| Belcheva et al. (2014) | Illumina MiSeq | Colon tissue samples | • Microbial-derived butyrate induces the hyperproliferation of colonocytes in an $MSH2^{-/-}$ mice model<br>• Gut microbiota did not drive CRC via an inflammatory pathway or genotoxic mechanisms |

| | | | |
|---|---|---|---|
| Zackular et al. (2013) | Roche/454 GS-FLX Titanium (pyrosequencing) | Feces | • OTUs associated with *Bacteroides*, *Odoribacter*, and *Akkermansia* were found to be enriched and those associated with *Prevotellaceae* and *Porphyromonadaceae* decreased in a CRC murine model<br>• Germ-free mice who received microbiota from tumor-bearing mice were found to have significantly increased colorectal carcinogenesis compared with mice colonized with a healthy gut microbiome<br>• Treatment with antibiotics decreased dramatically the tumor number and size |
| Wu et al. (2013) | Roche/454 GS-FLX (pyrosequencing) | Feces | • V3 region of 16S rRNA gene pyrosequencing demonstrated a strong difference in fecal microbiota of CRC and healthy individuals<br>• Microbiota of CRC subjects were enriched by Bacteroides, whereas abundance of OTUs affiliated with butyrate-producing genera *Faecalibacterium* and *Roseburia* was reduced<br>• Potentially tumorigenic *Fusobacterium* and *Campylobacter* species were significantly more abundant.<br>• Bacteroides species were positively correlated with tumor status |
| Warren et al. (2013) | Illumina MiSeq and Illumina HiSeq 2000 | Tumor samples | • Anaerobe bacteria including *Fusobacterium*, *Leptotrichia,* and *Campylobacter* species were significantly co-occurred with CRC<br>• This bacterial co-occurrence was found associated with overexpressed host pro-inflammatory-encoded gene IL-8 |
| Geng et al. (2013) | Roche/454 GS-FLX Titanium (pyrosequencing) | Tumor and normal tissue samples | • Based on 16S rRNA gene pyrosequencing, Chinese CRC patients had an increased tumor-associated bacterial diversity |
| Ahn et al. (2013) | Roche/454 GS-FLX Titanium (pyrosequencing) | Feces | • *Fusobacterium* and *Porphyromonas* were found more abundant in CRC patients compared with healthy individuals |
| McCoy et al. (2013) | Roche/454 GS-FLX Titanium (pyrosequencing) | • Normal and adenomatous tissue samples<br>• Tumor tissues and adjacent normal mucosa | • Individuals with high *Fusobacterium* abundance were significantly more likely to have adenomas<br>• Local cytokine gene expression in CRC subjects had a positively significant correlation with abundance of *Fusobacterium* |

(continued)

**Table 5.1** (continued)

| Author/Year | Sequencing technology | Sampling | Findings |
|---|---|---|---|
| Chen et al. (2013) | Roche/454 GS-FLX Titanium (pyrosequencing) | Feces | • Compared with healthy individuals, *Enterococcus* and *Streptococcus spp.* were significantly more prevalent in advanced colorectal adenoma |
| Brim et al. (2013) | Roche/454 GS-FLX Titanium (pyrosequencing) | Feces | • Pyrosequencing of fecal microbiota samples of subjects with colon polyps did not show major differences compared with controls |

*CMS1* consensus molecular subtypes 1, *CRC* colorectal cancer, *FOBT* fecal occult blood test, *IL-8* interleukin 8, *MSH2* mutS homolog 2, *NGS* next-generation sequencing, *OTUs* operational taxonomic units, *rRNA* ribosomal RNA, *TH1* T helper 1, *TH17* T helper 17

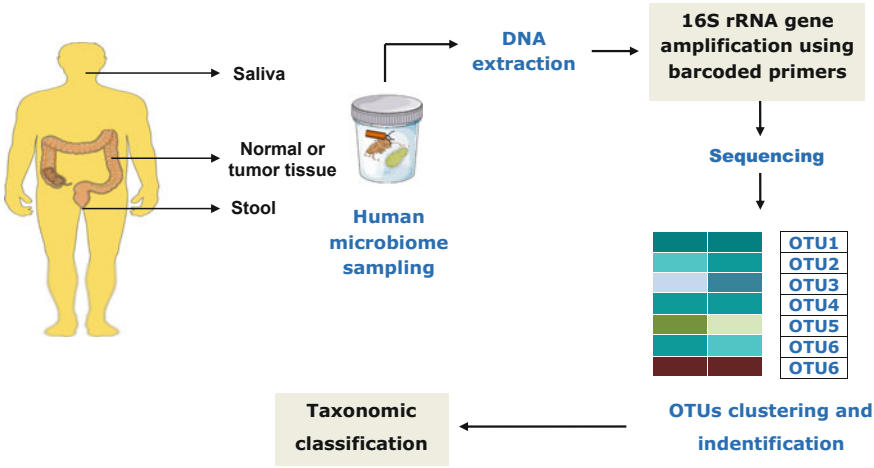



**Fig. 5.4** Metagenomic strategy used for the identification of bacterial communities associated with the host. For comments, see text. *DNA* deoxyribonucleic acid, *OTU* operational taxonomic unit

the studies investigating CRC-associated microbiome, 16S rRNA gene-targeted sequencing is being extensively used as a cheap and reliable phylogenetic marker to study the bacterial diversity in feces, saliva, and normal or tumor samples (Clavel et al. 2016; Morgan and Huttenhower 2012). After sequencing, one of the metagenomic strategies used to identify bacterial communities is based on the comparison of similar or nearly identical gene sequences and then assembled into clusters known as operational taxonomic units (OTUs) affiliated with close bacterial populations using phylogenetic and computational tools (Fig. 5.4) (Morgan and Huttenhower 2012). In an Illumina MiSeq-based study, Peters et al. enrolled 540 individuals screened for CRC using colonoscopy including subjects with conventional adenomas, serrated polyps, and controls and compared their associated gut microbiota diversity and abundance by sequencing their fecal 16S rRNA gene (Peters et al. 2016). In this large study, the diversity of gut microbiota was found decreased in adults with conventional adenoma-associated CRC compared to controls. Furthermore, subjects with advanced stages of adenoma had more reduced community diversity which underscores a possible role of microbiota in the adenoma-carcinoma sequence (Peters et al. 2016). A metatranscriptomic analysis based on Illumina HiSeq 2000 platform indicated that tissues from CRC patients are enriched with co-occurred anaerobe bacteria including *Campylobacter*, *Leptotrichia*, and *Fusobacterium* (Warren et al. 2013). This last genus is widely studied in the previous literature using NGS platforms, and its abundance was found significantly altered in CRC (Castellarin et al. 2012; Kostic et al. 2012; Rubinstein et al. 2013; McCoy et al. 2013; Ahn et al. 2013; Wu et al. 2013; Mira-Pascual et al. 2015; Yang et al. 2017b; Mehta et al. 2017; for review: Bashir et al. 2015; Gholizadeh et al. 2017); it was also found associated with chemoresistance by modulation of autophagy (Yu et al. 2017b). Notably, this bacterial co-occurrence

was associated with overexpressed host pro-inflammatory-encoded gene IL-8 (Warren et al. 2013). Flemer et al. evaluated microbiota composition subjects with adenoma, CRC subjects, and healthy individuals prospectively by sequencing 16S rRNA gene (Flemer et al. 2017a). As noted in previous reports, microbiota profile of CRC subjects varies from that of healthy controls as well as between distal and proximal CRC (Flemer et al. 2017a). Accordingly, OTU data showed an increase in the abundance of *Bacteroidetes* Cluster 2, *Firmicutes* Cluster 2, Pathogen Cluster, and *Prevotella* Cluster in tumor biopsy, but also chemokine (C-X-C motif) ligand 1 (CXCL1), serpin family E member 1 (SERPINE-1), signal transducer and activator of transcription 3 (STAT3), and interleukin- (IL-17 and IL-23) related genes were found positively correlated with this microbial signature, all known for their involvement in a pro-inflammatory and hostile niche (Flemer et al. 2017a). Again, in another report profiling gut microbiota in CRC patients using pyrosequencing (Roche/454 GS-FLX), Gao et al. found that abundance of *Fusobacterium* and *Lactococcus* is relatively increased in tumor samples compared with normal adjacent tissues (Gao et al. 2015). According to tumor location, this study also suggested that some potential pro-oncogenic bacteria are differentially abundant and indicated that distal CRC is relatively enriched by *Fusobacterium*, *Escherichia-Shigella*, and *Leptotrichia* compared to proximal tumor tissue samples (Gao et al. 2015). Moreover, an Illumina MiSeq-based 16S rRNA gene sequencing of colonic and fecal microbiota confirmed these previous results and found an enrichment of CRC tissues by *Fusobacterium*, *Selenomonas*, and *Peptostreptococcus* (Hibberd et al. 2017). Interestingly, patients who received a probiotics treatment with daily tablets of *Bifidobacterium lactis* Bl-04 (1.4 × 1010 CFUs) and *Lactobacillus acidophilus* NCFM (7 × 109 CFUs) had reduced *Fusobacterium* and *Peptostreptococcus* in fecal samples which may have therapeutic intervention perspectives and supports the fact that gut microbiota associated with CRC is potentially modifiable (Hibberd et al. 2017).

Experimentally, supportive and unequivocal evidence from in vivo studies has also emerged to deeply understand the role of gut microbiome in CRC. In a recent *Cell* report, Belcheva et al. used an $APC^{Min/+}$ $MSH^{(-/-)}$ murine model developing spontaneously multiple and numerous intestinal adenomas to examine the effects of gut microbiota metabolism on the colon tissue (Belcheva et al. 2014). Unexpectedly, carbohydrate-derived oncometabolites such as butyrate were found to drive CRC by inducing aberrant proliferation of colon epithelial cells (Belcheva et al. 2014; Donohoe et al. 2012). Butyrate is a short-chain fatty acid obtained from anaerobic bacterial fermentation such as of dietary fibers by *Firmicutes* and has been shown previously to have anticancer effects by inhibiting histone deacetylases (HDACs) (Encarnação et al. 2015; Singh et al. 2014; Gonçalves and Martel 2013). Excitingly, antibiotic treatment at 6 weeks of age decreased significantly the polyp number in this model which suggests that diet containing low-carbohydrates may prevent the transformation of polyps in hereditary predisposed subjects (Belcheva et al. 2014). Two other reports by Zackular et al. explored the roles of microbiota in colorectal carcinogenesis in mice induced by intraperitoneal injection of azoxymethane (Zackular et al. 2013, 2016). Gut microbiome (especially *Bacteroides*) exacerbated CRC formation in this model alongside with a successful reduction in tumor burden

after treatment by antibiotics (Zackular et al. 2013). In addition, microbiota transfer from tumor-bearing mice to germ-free animals significantly increased the tumor number and size compared to controls (Zackular et al. 2013). Later, Zackular's team also investigated the potential of microbiome perturbation by antibiotics cocktails in a similar murine model and found that treatment during different colorectal carcinogenesis stages could stop the occurrence of additional tumors (Zackular et al. 2016). Another interesting study aimed to see whether feeding stool of CRC patients to germ-free and azoxymethane-treated conventional mice will drive tumorigenesis (Wong et al. 2017). 16S rRNA gene sequencing based on Illumina MiSeq showed many abundant pro-tumorigenic bacteria such as *Bacteroides fragilis* and *Fusobacterium nucleatum* and an increase in the incidence of polyps, dysplasia, cellular proliferation, pro-inflammatory cytokines, and $T_H1/T_H17$ lymphocytes associated with colon tissues compared to controls (Wong et al. 2017), therefore providing evidence of the direct carcinogenic effects of microbiota in CRC.

Surprisingly, a very recent report in *Science* by Bullman et al. found that CRC cells metastasize hand in hand with gut microorganisms which will move this field toward other novel unexpected findings in the near future (Bullman et al. 2017; Yang and Jobin 2018). The Bullman's study has shown that *Fusobacterium nucleatum* and other CRC-associated microbiome are maintained in distal metastatic tumors (Bullman et al. 2017). Based on in situ hybridization (ISH) and NGS platforms including Illumina HiSeq2500 and Illumina MiSeq, this study revealed that primary and metastatic colorectal tumor pairs harbor high levels of similar strains of *Fusobacterium nucleatum* (>99.9% average nucleotide identity) (Bullman et al. 2017). Likewise, 16S rRNA gene sequencing and RNA-Seq showed that in addition to the previously discussed CRC-associated bacteria, anaerobes including *Bacteroides fragilis*, *Bacteroides thetaiotaomicron*, *Prevotella intermedia*, and *Selenomonas sputigena* were also found to persist in matched metastatic tumors (Bullman et al. 2017). Strikingly, CRC patient's derived mouse xenografts retained viable microbiome and were successfully reduced when treated by metronidazole and a parallel decrease in tumor growth was also noted (Bullman et al. 2017). Therefore, tumor-associated gut microbiota is at the moment one other factor added to the catalogue of known risk disease co-determinants of CRC to be considered in further research.

## 5.4 Gut Microbiota as a Biomarker

In the context of biomarkers discovery, Zeller et al. investigated fecal microbiota as an early detection marker for CRC in a series of 156 patients utilizing metagenomic Illumina HiSeq-based whole-genome shotgun sequencing and Illumina MiSeq-based 16S rRNA gene sequencing approaches (Zeller et al. 2014) (Box 5.2). Changes in gene and species markers were noted during early stage of CRC and metagenomic detection accuracy was found similar to the standard validated screening with the fecal occult blood testing (FOBT) (Zeller et al. 2014). Potentially, a significant improvement in terms of accuracy (area under curve (AUC): 0.87) was

demonstrated using ROC curves when fecal microbial abundance and FOBT were combined (Zeller et al. 2014). Similarly, sequencing of V4 region of the 16S rRNA gene using Illumina MiSeq from stools of three clinically different groups including healthy subjects and patients with adenoma or carcinoma in combination with already known clinical risk factors such as age, race, and body mass index provided an improved screening ability to distinguish between these groups (Zackular et al. 2014). Recently, Flemer et al. profiled microbiota in fecal, mucosal, and oral swab samples of subjects with CRC, adenoma, and healthy controls and sequenced their related 16S rRNA using Illumina MiSeq (Flemer et al. 2017b). When combining oral and fecal microbiota OTUs, enhanced values of AUC for detecting adenomas and colorectal tumors were 0.98 (95%, CI: 0.95–0.98; sensitivity: 88%) and 0.94 (95%, CI: 0.87–0.94; sensitivity: 76%), respectively, as well as an improved specificity of 95% for both; thus, the inclusion of oral microbiota is suitable in future diagnostic studies (Flemer et al. 2017b) (for further reading, see Narayanan et al. 2014). Another interesting recent study used Illumina MiSeq platform to assess the microbiota, this time, as a candidate prognostic biomarker for CRC (Wei et al. 2016). Kaplan–Meier and Cox estimation models indicated that CRC patients with highly abundant *Bacteroides fragilis* and *Fusobacterium nucleatum* had poor survival (Wei et al. 2016). Furthermore, remarkable expression of BRAF and KRAS was found in the group of patients with high level of *Fusobacterium nucleatum* and *Bacteroides fragilis* (Wei et al. 2016). Further analysis using over 1000 CRC cases from prospective cohort studies has drawn a similar conclusion supporting the hypothesis that tissue infiltrates by *Fusobacterium nucleatum* are associated with shorter patient's survival (Mima et al. 2016). Collectively, these optimistic advances are further supported by recent meta-analytic systematic reviews (summarized in Table 5.2), which are particularly valuable in microbiome research as they can provide an idea about reproducibility and evidence generating by pooling multiple studies. In this perspective, two recently and rigorously performed meta-analyses assessed the diagnostic accuracy of *Fusobacterium nucleatum* in detecting CRC (Peng et al. 2018; Huang et al. 2018). Huang et al. analyzed six case–control reports with more than 550 CRC cases and 700 healthy controls from Japan, Germany, Brazil, Sweden, and China and provided preliminary evidence of accuracy of using fecal fusobacteria as a diagnostic tool (Huang et al. 2018). Pooled overall specificity and sensitivity at 95% CI were 0.78 and 0.68, respectively, with an AUC of 0.8 (Huang et al. 2018). Furthermore, another systematic review that enrolled ten studies and 1198 subjects (629 patients and 569 controls) found that analysis of fecal and tissue *Fusobacterium nucleatum* may detect CRC with a sensitivity of 0.81 (95% CI: 0.64–0.91) and specificity of 0.77 (95% CI: 0.59–0.89) as well as an AUC of 0.86 (95% CI: 0.83–0.89) (Peng et al. 2018). Based on GRADE approach (Guyatt et al. 2011) (https://gradepro.org/), there is a moderate level of evidence to support the use of this signature in clinical practice (Huang et al. 2018). However, substantial heterogeneity (>80%) in these meta-analyses was observed due to underpowered sample sizes and differences in DNA extraction methods across studies and geographical regions of study participants (Zhang et al. 2019; Amitay et al. 2018; Huang et al. 2018; Peng et al. 2018). Therefore, confirmatory findings from large clinical

**Table 5.2** Summary of systematic reviews and meta-analyses evaluating the diagnostic role of *Fusobacterium nucleatum* in colorectal cancer

| Author/ Year | *N* | Number of included patients | Accuracy (95% CI) | Heterogeneity ($I^2$) present? | Reporting guideline used? | QUADAS-2 used? | NOS used? | SGA provided? |
|---|---|---|---|---|---|---|---|---|
| Zhang et al. (2019) | 10 | • Colorectal cancer:<br>Cases: 1450<br>Controls: 1421<br>• Colorectal adenoma:<br>Cases: 656<br>Controls: 827 | • Colorectal cancer<br>Sp: 76% (66%–84%)<br>Sn: 71% (61%–79%)<br>AUC: 0.80 (0.76–0.83)<br>• Colorectal adenoma<br>Sp: 73% (65%–79%)<br>Sn: 36% (27%–46%)<br>AUC: 0.60 (0.56–0.65) | Yes (substantial) | No | Yes | No | Yes (incomplete) |
| Amitay et al. (2018) | 19 | Colorectal cancer<br>Cases: 755<br>Colorectal adenomas: 772<br>Controls: 1477 | AUC: 0.68–0.77 | Not assessed (qualitative synthesis) | Yes (PRISMA) | No | Yes | No |
| Huang et al. (2018) | 6 | Cases:557<br>Controls:704 | Sp: 0.78 (0.75–0.81)<br>Sn: 0.68 (0.64–0.72)<br>AUC: 0.80.<br>DOR: 8.75 (4.86–15.78) | Yes (substantial) | Yes (PRISMA) | Yes | No | Yes |
| Peng et al. (2018) | 7 | Cases: 629<br>Controls: 569 | Sn: 0.81 (0.64–0.91) Sp: 0.77 (0.59–0.89) AUC: 0.86 (0.83–0.89)<br>DOR: 14.00 (9.00–22.00) | Yes (substantial) | Yes (MOOSE) | Yes | No | Yes |

*AUC* area under the curve, *DOR* diagnostic odds ratio, $I^2$ test of heterogeneity, *N* number of enrolled studies, *MOOSE* Meta-Analysis of Observational Studies in Epidemiology, *NOS* Newcastle-Ottawa Scale, *PRISMA* preferred reporting items for systematic reviews and meta-analyses, *QUADAS-2* Quality Assessment of Diagnostic Accuracy Studies, *SGA* subgroup analysis, *Sp* specificity, *Sn* sensitivity

trials are needed in the future to progress the ability of this noninvasive biomarker for CRC screening. Notably, in an important recent advance from the German Zeller' team, Wirbel et al. meta-analyzed eight robust reports ($n = 768$) that used fecal shotgun sequencing method to study CRC-associated microbiome changes with a particular focus on quantification of confounding effect caused by demographic patients' characteristics and technical aspects (Wirbel et al. 2019). Among 849 species detected across included studies, the authors identified a signature of 29 bacterial species including the prominent *Fusobacterium nucleatum* that were significantly enriched in CRC and undetectable in normal tissues that can be used to develop biomarkers for diagnosis (Wirbel et al. 2019). Moreover, functional microbiome analysis suggested that sequenced metagenomes were enriched by genes linked to protein-mucin catabolism as compared with a depletion of genes associated with carbohydrate degradation (Wirbel et al. 2019). Together, these findings and other similar recent progress (Thomas et al. 2019) will enable accurate designs of disease-predictive molecular diagnostic assays to be used in large clinical trials to aid in the diagnostic workflow of CRC and therefore to improve clinical outcomes.

## 5.5 Gut Microbiota and Cancer Immunotherapy

Tremendous success has been achieved during the last decade in improving survival outcomes of aggressive cancers such as metastatic melanoma and lung cancer as a consequence of the emergence of ICI (Pyo and Kang 2017; Yun et al. 2016; Herzberg et al. 2016; Tan et al. 2018) with distinct cellular mechanisms (Wei et al. 2017). Cancer cells escape from immunosurveillance by inactivating TILs including $CD4^+$ and $CD8^+$ T cells (Ganesh et al. 2019) (Fig. 5.5). This tumor property is known as "evading immune destruction" which is a cancer hallmark (Hanahan and Weinberg 2011). Immune checkpoints include CTLA-4 and PD-1/PD-L1 axis and are involved in the basic homeostatic negative feedback of T-cell activation after completing their immune functions (Sharma and Allison 2015). The human tumor texture is characterized by a mixture of cell types including tumor cells, immune cells (T cells, dendritic cells, NK cells, etc.), as well as fibroblasts and other components which constitute a complex cross-talk between cancer and the surrounding microenvironment. Basically, T-cell activation requires recognition of tumor antigens (antigenic epitopes) by major histocompatibility complex (MHC), and then, they proliferate and differentiate into anticancer effector cells (Sharma and Allison 2015). On the other hand, additional co-stimulatory and determinant signals such as B7 and CD28 are needed to activate naïve T cells which infiltrate the target tumors (TILs). Notably, inside the tumor stroma, activated immune cells have to surmount additional barriers including inhibitory cytokines and immunosuppressive FOXP3 $T^{reg}$ cells (Najafi et al. 2019; Takeuchi and Nishikawa 2016; Sharma and Allison 2015). Activated T cells induce CTLA-4 expression which binds to B7 and accumulates leading to immune response abrogation (Sharma and Allison 2015). Furthermore, PD-1 immune-checkpoint expression is another player in T-cell response blockade that binds to tumor cell ligands PD-L1 (or PD-L2) and leads to

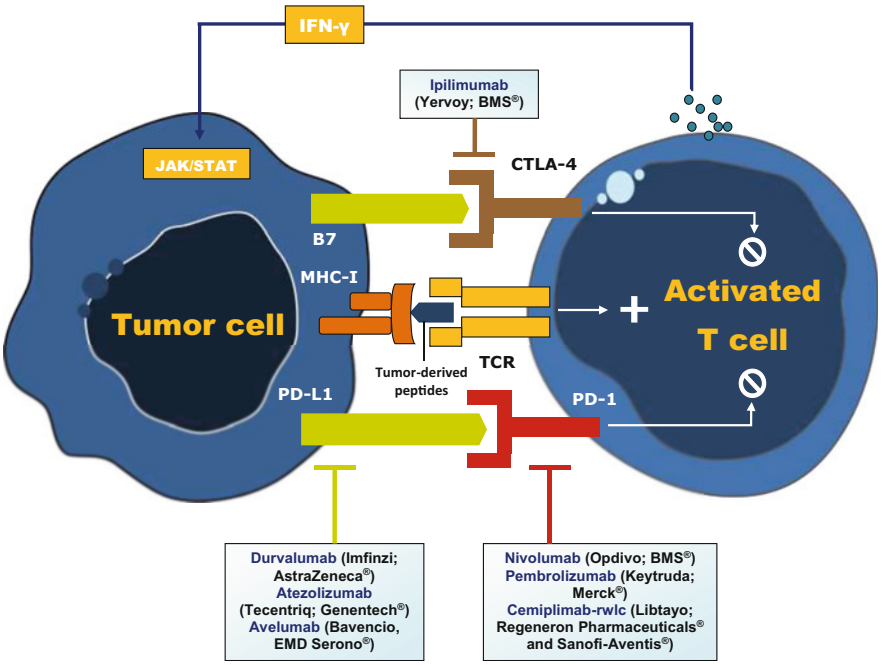



**Fig. 5.5** Cancer cell escape from immunosurveillance. For comments, see text. *CTLA-4* cytotoxic T-lymphocyte antigen 4, *IFN-γ* interferon gamma, *JAK* Janus kinase, *MHC-I* major histocompatibility complex I, *PD-1* programmed cell death 1, *PD-L1* programmed death-ligand 1, *STAT* signal transducers and activators of transcription, *TCR* T cell receptor

anergy or T-cell death (Ganesh et al. 2019; Sharma and Allison 2015). However, this topic is more complex than discussed in this chapter and additional specialized reading about deep signaling in tumor immunology is recommended (for review, see Topalian et al. 2015; Pardoll 2012).

According to various prognostic CRC studies, TILs are predictive of improved survival (Nazemalhosseini-Mojarad et al. 2019; Kong et al. 2019; Rozek et al. 2016; Mei et al. 2014). Remarkably, recent data suggest that—in addition to tumor mutational burden (Keenan et al. 2019; Ganesh et al. 2019)—gut microbiota may influence the efficacy of immune-checkpoint blockade as well as the occurrence of related digestive toxicities such as colitis by modulating host immunity (Pitt et al. 2016; Murphy et al. 2019; Havel et al. 2019; Li et al. 2019). In this perspective, several lines of evidence particularly from prospective studies (Table 5.3) have illuminated this potential connection. Response prediction to ICI beyond PD-1/PD-L1 and CTLA-4 expression is now revisited and other factors such as gut microbiota may be added to the current biomarkers used in practice. An earlier in vivo study aimed to investigate the underlying distinct response factors to ICI in mice with melanoma with different microbiota composition (Sivan et al. 2015). 16S rRNA sequencing categorized mice with abundant *Bifidobacterium* which were

**Table 5.3** Summary of emerging data linking gut microbiota and response to cancer immunotherapy

| Tumor (enrollment) | Study design | Microbiota study method | Immune checkpoint inhibitor | Findings | References |
|---|---|---|---|---|---|
| Stage IV colorectal cancer ($n = 80$) | Prospective | 16S rRNA sequencing | Pembrolizumab or other immune-checkpoint inhibitors (ICI) | This study is ongoing (estimated completion: April 2020) | NCT02960282 |
| Metastatic melanoma (112) | Prospective | 16S rRNA sequencing and Metagenomic shotgun sequencing (Illumina MiSeq and HiSeq) | PD-1 blockade (not specified) | • Responders to PD-1 blockade have significant differences in terms of gut microbiome composition and diversity as compared with nonresponders<br>• Abundance of Ruminococcaceae bacteria was significantly higher ($p < 0.01$) in the responding patients | Gopalakrishnan et al. (2018) |
| Epithelial cancers ($n = 249$)[a] | Retrospective + in vivo | Metagenomic shotgun sequencing (Ion Proton) | PD-1/PD-L1 blockade | • Treatment by antibiotics decreased the efficacy of immune-checkpoint blockade in patients with advanced cancers<br>• Transplantation of fecal microbiota from responders to anti-PD-1 into germ-free improved the anticancer effects immunotherapy<br>• A correlation between response to anti-PD-1 monoclonal antibodies and the relative abundance of *Akkermansia muciniphila* was noted | Routy et al. (2018b) |
| Metastatic melanoma (26) | Prospective | 16S rRNA sequencing (454 pyrosequencing and Illumina MiSeq) | Ipilimumab | • Improved progression-free survival ($p = 0.0039$) and overall survival ($p = 0.051$) were observed in melanoma patients with enriched microbiota by *Faecalibacterium* genus and other Firmicutes<br>• Immunotherapy-induced colitis was seen in patients with baseline phylotypes enriched by Firmicutes as compared with patients with *Bacteroidetes* (no colitis) | Chaput et al. (2017) |

| | | | | | |
|---|---|---|---|---|---|
| Metastatic melanoma (39) | Prospective | Metagenomic shotgun sequencing (Illumina HiSeq) | Pembrolizumab, ipilimumab, nivolumab, or ipilimumab + nivolumab | • Improved response to ICI was seen in patients with enriched microbiota by *Bacteroides caccae*<br>• Microbiota of responders treated with ICI was enriched by *Faecalibacterium prausnitzii, Bacteroides thetaiotaomicron*, and *Holdemania filiformis*<br>• Responders to pembrolizumab had enriched microbiota by *Dorea formicogenerans* | Frankel et al. (2017) |
| Metastatic melanoma (34) | Prospective | 16S rRNA sequencing (Illumina MiSeq) | Ipilimumab | • Abundant fecal *Bacteroidetes* phylum and microbial genetic signaling pathways related to transport of polyamine and biosynthesis of B vitamin correlate with resistance to CTLA-4 blockade-induced colitis | Dubin et al. (2016) |
| Melanoma | Preclinical (mice) | 16S rRNA sequencing (Illumina MiSeq) | PD-L1 mAb therapy | • Therapeutic intervention by oral administration of *Bifidobacterium* alone or in combination with immune-checkpoint blockade enhanced antitumor control<br>• This anticancer effect is mediated by the $T_{CD8+}$ cell tumor microenvironment | Sivan et al. (2015) |

[a]Renal cell carcinoma ($n = 67$), advanced non-small cell lung cancer ($n = 140$), and urothelial carcinoma ($n = 42$)

associated with notable antitumor effects (Sivan et al. 2015). Interestingly, oral administration of *Bifidobacterium* displayed significant antitumor activity as compared with mice controls. When combined with anti-PD-L1 antibody, this anti-melanoma property was found to suppress tumor outgrowth (Sivan et al. 2015). Mechanistically, it was seen that this synergistic effect was associated with remarkable $CD8^+$ T-cell priming and accumulation in tumor stroma (Sivan et al. 2015). Recently, 39 metastatic melanoma patients were enrolled in a prospective manner to identify those who may benefit from ICI (ipilimumab, nivolumab, ipilimumab + nivolumab, or pembrolizumab) based on shotgun sequencing of their gut microbiota (Frankel et al. 2017). *Bacteroides caccae* was found enriched in all ICI responders (Frankel et al. 2017). In addition, patients treated with ipilimumab + nivolumab had abundant *Faecalibacterium prausnitzii*, *Bacteroides thetaiotaomicron*, and *Holdemania filiformis* (Frankel et al. 2017). In the pembrolizumab responders' cohort, *Dorea formicogenerans* was found enriched compared with patients who had progressive disease under ICI (Frankel et al. 2017). Importantly, gut metabolomic profiling identified high levels of the natural product anacardic acid in the responders to ICI which suggests its potential as a future predictive biomarker for this setting (Frankel et al. 2017). Of note, anacardic acid is a xenobiotic natural compound known to induce macrophage and neutrophil activation (Hollands et al. 2016; Gnanaprakasam et al. 2015) and therefore enhance adaptive immune system and ICI. More recently and based on a prospective cohort of patients with advanced melanoma ($n = 26$) and treated with anti-CTLA-4 ipilimumab, Chaput et al. showed that patients with improved progression-free survival and overall survival ($p = 0.0039$ and $p = 0.051$, respectively) had enriched gut microbiota by *Faecalibacterium* genus and other Firmicutes (cluster A) at baseline (Chaput et al. 2017). However, clinically meaningful response in this setting was limited by frequent occurrence of immune-related digestive toxicity including enterocolitis which was interestingly absent in patients with enriched microbiota by *Bacteroidetes* (Chaput et al. 2017). A further serum analysis of these patients found that treatment with ipilimumab increased circulating CD25 in the group with enriched *Faecalibacterium*-driven cluster A and thus a promising role for monitoring drug response to ICI (Chaput et al. 2017). Similarly, Gopalakrishnan et al. analyzed oral and gut microbiota of 112 melanoma patients and observed significant differences in terms of composition and diversity in the responding group compared with the resistant group treated with anti-PD-1 immunotherapy (Gopalakrishnan et al. 2018). Significantly higher alpha diversity and relative abundance of Ruminococcaceae bacteria were noticed in fecal samples of 34 patients (30 responders and 13 nonresponders; $p < 0.01$ for both) (Gopalakrishnan et al. 2018). Based on metagenomic sequencing, microbiota of responders was enriched by anabolic pathways. Moreover, enhanced antitumor activity of anti-PD-1 therapy seen in the responding cohort may be attributed to higher intratumor antigen presentation and T-cell functions (higher density of $CD8^+$ T infiltrates) as compared with melanoma patients that were resistant and in which there was a limited immune cell infiltration (Gopalakrishnan et al. 2018). Importantly, flow cytometry analysis showed a systemic immune response by increased levels of effector $CD4^+/CD8^+$ T

lymphocytes as well as preserved cytokine response in patients with highly abundant gut Clostridiales, Ruminococcaceae, or *Faecalibacterium* compared with patients with enriched bacteroidales in which suppressive cells including $T^{reg}$ lymphocytes and myeloid-derived suppressor cells were higher (Gopalakrishnan et al. 2018). In view of these findings, favorable gut microbiota in cancer patients may improve the human profile of the host and thus boost the efficacy of cancer immunotherapy. Confirmatory results from a large cohort of 249 patients with epithelial cancers allocated to PD-1/PD-L1 inhibitors demonstrated that treatment by antibiotics was correlated with a decreased response to these agents (Routy et al. 2018b). It seems from this study that response to anti-PD-1 antibodies is associated with relatively abundant *Akkermansia muciniphila*, which was also confirmed when this bacterium was orally supplemented to antibiotic-treated mice and a significant restoration of PD-1 blockade was noticed (Routy et al. 2018b). Interestingly, antibiotics intake by melanoma patients within 30 days prior to ICI initiation was found recently as an adverse effect of progression-free survival (HR: 0.32; 95% CI: 0.13–0.83; $p = 0.02$) (Elkrief et al. 2019). In other advanced solid cancers, gut alteration by antibiotics treatment leads to dysbiosis and affects the effectiveness of immune-checkpoint blockade (Zhao et al. 2019; Derosa et al. 2018b; Ahmed et al. 2018) and showed a tendency to short survival. In CRC, there is an ongoing prospective clinical trial (NCT02960282) conducted by the University of Southern California in collaboration with the National Cancer Institute (NCI) and will assess gut microbiome a predictive biomarker for chemotherapy (FOLFOX or FOLFIRI) or immunotherapy (pembrolizumab or other ICI) in the metastatic setting. This clinical trial is currently recruiting patients for which fecal specimens will be collected at baseline before and at various stages of treatment courses as well as during disease progression. 16S rRNA sequencing will be performed in addition to transcriptomic and metaproteomic analyses.

Immune-checkpoint blockade has become a part of the standard of care for dozens of different cancers as monotherapy or in combination with other anticancer drugs (Fig. 5.6). Despite being immature, the accumulating evidence supports the concept that response to cancer immunotherapy is microbiota dependent (Gong et al. 2019). However, research on this hot topic is at the beginning, and therefore, additional in vivo and human clinical trials are needed to profoundly understand the role of gut microbiota in predicting outcomes of immune-checkpoint blockade. Unanswered questions have to be carefully examined before implementing these advances in the practice of clinicians. First, (I) 16S rRNA sequencing approach is the most used in metagenomic projects of gut microbiota until this time despite it is associated with potential biases introduced during the PCR amplification step, and therefore, this concern should be considered and improved in the development of future NGS accurate platforms. In addition, (II) we currently do not know if these described bacterial communities and species are accurate predictive biomarkers for ICI because of the small sample size of the conducted studies until this time. Importantly, (III) exposure and timing of antibiotics and stool analysis before ICI treatment need to be studied deeply to see whether it is adequate or it requires other circulating biomarkers for response prediction. And finally, (IV) long-term

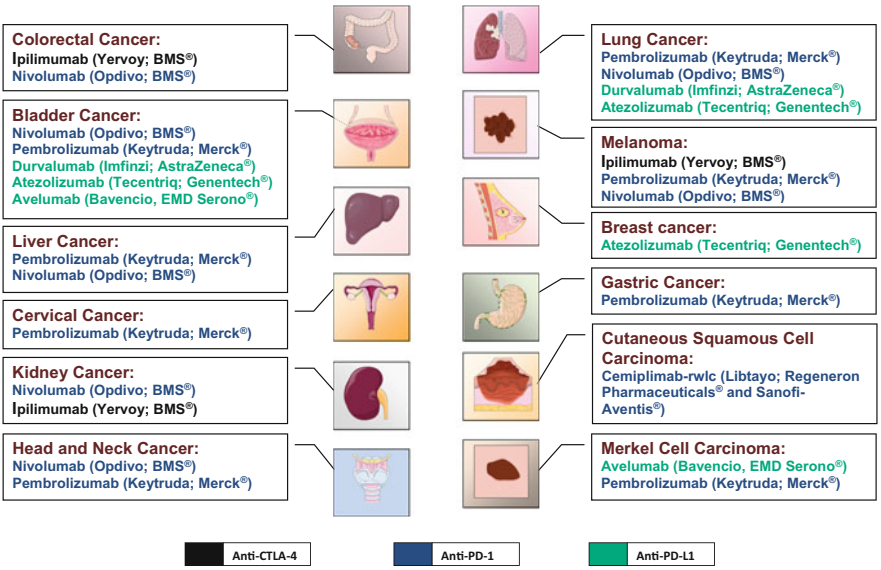



**Fig. 5.6** General overview of FDA-approved and currently available immune-checkpoint inhibitors for clinical use in solid cancers

remissions were seen in a subset of cancer patients which strongly support the current interest in developing predictive biomarkers including microbiota for patients' selection and stratification. Therefore, future biomarker-driven clinical studies using ICI should consider this progress in their study design especially basket trials.

## 5.6 Conclusion and Perspectives

On the basis of these data discussed here, there is unequivocal evidence to illustrate the association of gut microbiome dysbiosis with CRC and its possible future use as a biomarker, but—due to the small sample size—further evaluations are required especially in the context of clinical trials. Promisingly, nine observational studies with a prospective design investigating gut microbiota and CRC based on predominantly 16S rRNA sequencing approach may provide additional data to the current knowledge on this topic (summarized in Table 5.4). Solid cancers—and CRC in particular—are genetic diseases which support the hypothesis of microbiota-driven carcinogenesis through its direct or indirect roles in inducing DNA damages and sustained tumor proliferation. Notably, ICI represent a radical change in the management of advanced cancers but are currently facing unique challenges especially the development of acquired resistance (Gide et al. 2017; Syn et al. 2017). Therefore, panels of biomarkers that take into consideration microbiota (specific bacterial signatures for example) to guide ICI in drug development and to maximize their

**Table 5.4** Observational prospective clinical trials investigating gut microbiota and colorectal cancer based on 16S rRNA sequencing or other methods

| Clinical trials identifier | Study design/ Current status/ Estimated completion date | Setting/Enrollment | Interventions/Biospecimens/Study methods | Sponsor |
|---|---|---|---|---|
| NCT02960282 | • Prospective<br>• Recruiting<br>• April 2020 | Stage IV colorectal cancer (CRC) ($n = 80$) | • Group 1: Patients who will initiate first-line FOLFOX- or FOLFIRI-based therapy<br>• Group 2: Patients who will initiate Pembrolizumab (or another immune-checkpoint inhibitor) as monotherapy<br>• Fecal specimens<br>• 16S rRNA sequencing | University of Southern California in collaboration with National Cancer Institute (NCI) |
| NCT03843905 (METABIOTE) | • Prospective<br>• Recruiting<br>• November 2021 | Sporadic CRC ($n = 300$) | • Prognostic impact of gut and tumor-associated microbiota on surgical and oncologic outcomes of patients who underwent surgery<br>• Microbiota composition according to sampling sites<br>• Fecal, peritumoral mucosa, and tumor samples<br>• 16S rRNA sequencing | University Hospital, Clermont-Ferrand in collaboration with M2iSH laboratory and Benoit Chassaing |
| NCT02371135 | • Prospective<br>• Recruiting<br>• February 2020 | Lynch Syndrome and other hereditary colonic polyposis syndromes ($n = 225$) | • Association of gut microbiome and dietary factors with risk of adenoma or cancer in patients with Lynch and other hereditary syndromes.<br>• Colonic biopsies<br>• 16S rRNA sequencing | Memorial Sloan Kettering Cancer Center in collaboration with Harvard University<br>Broad Institute |
| NCT02141945 | • Prospective<br>• Completed<br>• March 2017 | Subjects undergoing polyp surveillance or screening by colonoscopy (ASA Class 1–3) ($n = 260$) | • Study of the accuracy and accuracy of the Metabiomics Colon Polyp and Colorectal Cancer Assay for colon polyps or CRC detection compared with pathologic and endoscopical results<br>• Fecal, rectal, and colonic mucosa samples<br>• Metabiomics Colon Polyp and Colorectal Cancer Assay | Metabiomics Corp |
| NCT02151123 | • Cross-sectional<br>• Not yet recruiting<br>• September 2020 | Patients undergoing surgery for CRC ($n = 100$) | • Determination of the percentage of false-negative results of Metabiomics Colon Polyp and Colorectal Cancer Assay<br>• Fecal rectal and colonic mucosa samples<br>• Metabiomics Colon Polyp and Colorectal Cancer Assay | Metabiomics Corp |

(continued)

**Table 5.4** (continued)

| Clinical trials identifier | Study design/ Current status/ Estimated completion date | Setting/Enrollment | Interventions/Biospecimens/Study methods | Sponsor |
|---|---|---|---|---|
| NCT02947607 | • Prospective<br>• Recruiting | • Subjects undergoing lower endoscopy without adenoma or CRC<br>• Patients with detected adenoma on lower endoscopy<br>• Patients with resected CRC ($n = 1125$) | • Study of differential host microbiome composition and abundance in healthy subjects, and patients with adenoma and CRC as well as its correlation with host features.<br>• Saliva, stool, and colon biopsy specimens<br>• 16S rRNA sequencing | Universitaire Ziekenhuizen Leuven in collaboration with Janssen Pharmaceutica N. V., Belgium and KU Leuven |
| NCT02726243 (DYSCOLIC) | • Prospective<br>• Recruiting<br>• August 2020 | Subjects with CRC and inflammatory bowel disease (IBD) ($n = 240$) | • Study of the characteristics of gut microbiota in patients with CRC and IBD<br>• Fecal samples<br>• 16S rRNA sequencing (Illumina MiSeq) | Assistance Publique—Hôpitaux de Paris in collaboration with INSERM, France |
| NCT03297996 | • Prospective<br>• Completed<br>• April 2015 | Patients with serrated or hyperplastic polyps, sessile serrated adenoma and CRC ($n = 540$) | • Comparison between gut microbiota diversity and overall composition in subjects with preneoplastic lesions and CRC<br>• Fecal samples<br>• 16S rRNA sequencing (Mobio PowerSoil DNA Isolation Kit—Roche 454 FLX Titanium) | New York University School of Medicine |
| NCT03383159 | • Prospective<br>• Recruiting<br>• September 2020 | Patients with or without metachronous adenomas after colorectal surgery ($n = 100$) | • Construction of a predictive model of postoperative CRC occurrence based on the analysis of gut microbiota.<br>• Fecal samples<br>• 16S rRNA sequencing | First Affiliated Hospital of Harbin Medical University |

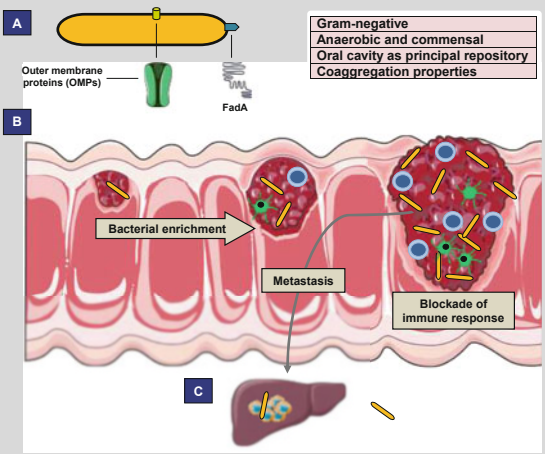
Data from ClinicalTrials.gov (accessed 05/04/2019)

*ASA* American Society of Anesthesiologists-physical status score, *FOLFIRI* Folinic acid (leucovorin) Fluorouracil (5-FU) irinotecan, *FOFLOX* Folinic acid (leucovorin) Fluorouracil (5-FU) Oxaliplatin, *rRNA* ribosomal RNA

effects are needed and are expected to achieve more accurate predictive models. The future of this field seems to be bright as uncultured and previously unknown human gut microbiota is now deciphered with an increase of phylogenetic diversity by 281% (Almeida et al. 2019). Predictive accuracy of studied gut microbial signatures was recently improved using cross-cohorts (Wirbel et al. 2019; Thomas et al. 2019) but needs to be validated in additional multicenter and independent well-powered cross-population studies.

Microbiota manipulation particularly fecal microbiota transplantation (Wu et al. 2019) from long-term responders to treatments seems to be promising as a therapeutic intervention for patients with CRC or those developing toxicities for ICI. In this perspective, this intervention has already demonstrated its effectiveness in treating recurrent *Clostridium difficile* infection (Staley et al. 2017) and ulcerative colitis (Ding et al. 2019; Paramsothy et al. 2017; Narula et al. 2017). "Good" and "bad" microbiota should be identified to provide more evidence for interventional procedures in the context of CRC or other diseases. However, remarkable concerns regarding the confounding effects influencing gut microbiome composition in these studies (antibiotics use for example) should be addressed in the future before implementing these advances in the clinic. Furthermore, the observed outcomes related to a particular associated microbiota have to be reproduced in additional well-designed studies and carefully controlled for confounding variables. This chapter sheds light on the potential of NGS technology in studying gut microbiota in CRC as well as its applications as a future biomarker for ICI. Recommended and additional reading about this complex topic can be found in Boxes 5.3 and 5.4.

### Box 5.1 Structure and Oncological Functions of *Fusobacterium nucleatum* in Colorectal Cancer

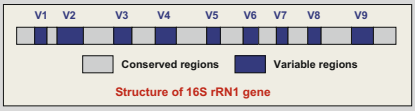
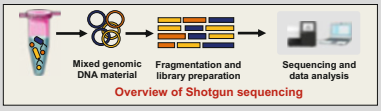



**A:** Structure of *Fusobacterium nucleatum* (FN). FN is characterized by OMPs and FadA implicated in coaggregation with other species and adherence respectively.

**B:** FadA bacterial antigen (adhesin) induces tumoral colonocytes proliferation by a mechanism involving E-cadherin/β-catenin signaling axis (Rubinstein et al. 2013). Bacterial infiltrates inhibit anticancer surveillance of myeloid cells and tumor-infiltrating lymphocytes. FN is associated with stages of colorectal carcinogesis including metastatic dissemination (Flanagan et al. 2014). (Adapted from: Brennan and Garrett, 2016).

**C:** Persistence of FN in metastatic colorectal tumors in the liver (according to Bullman et al. 2017).

## Box 5.2 Sequencing Methods for Gut Microbiota Analysis: 16S Ribosomal RNA Versus Shotgun Sequencing

| 16S rRNA sequencing | Shotgun sequencing |
|---|---|
| • A method based on targeted sequencing of the 16S ribosomal RNA bacterial gene.<br>• 16S rRNA gene contains conserved sequences and 9 hypervariable regions (V1–V9).<br>• Variation in V1-V9 sequences reflects evolutionary divergence and is widely used as a marker for bacterial identification, taxa analysis, and phylogenetic classification.<br>• Sequences are compared and aligned based on their similarity (OTU clustering) to provide taxonomy profiling according to the existing reference sequences.<br>• A relatively cheap method compared to whole-genome sequencing for studying "unculturable" gut bacteria.<br>• 16S rRNA sequencing data are relatively quantitative and are limited by the information publicly accessible in the reference databases.<br>• It cannot provide information related to microbiota functions and cause-and-effect relationships with the host.<br>• This method may be prone to biases because of PCR amplification and incompleteness of reference databases. | • Shotgun metagenomic techniques enable sequencing of all genomic materials present in a given sample in an unbiased manner as well as comprehensively whole-genome mapping of all genes in all microbes.<br>• Shotgun is based on random DNA fragmentation followed by sequencing of obtained small fragments from entire microbial communities.<br>• Shotgun metagenomic sequencing of microbial DNA provides superior deep understanding of community biodiversity and its functions.<br>• A time-consuming and expensive technique but with potential to study uncultured microbiota.<br>• Bioinformatic analysis of massive amounts of metagenomic data is complex and is a challenge for this method.<br>• Obtained data from shotgun sequencing are prone to contamination by the presence of unwanted host DNA. |
|  |  |

A practical step-by-step protocol for microbiota sequencing is nicely described by: Davidson and Epperson 2018 (doi: https://doi.org/10.1007/978-1-4939-7471-9_5) and Goodrich et al. 2014 (doi: https://doi.org/10.1016/j.cell.2014.06.037). Recommended reading for physicians: Sarangi et al. 2019 (doi:https://doi.org/10.1016/j.jceh.2018.04.016)

**Box 5.3 Recommended Articles of Particular Interest for English-Speaking Readers**

| | |
|---|---|
| Plottel CS, Blaser MJ. *Microbiome and malignancy*. Cell Host Microbe. 2011 Oct 20;10(4):324-35. | doi:https://doi.org/10.1016/j.chom.2011.10.003 |
| Schmidt TSB, et al. *The Human Gut Microbiome: From Association to Modulation*. Cell. 2018;172 (6):1198-1215. | doi:https://doi.org/10.1016/j.cell.2018.02.044 |
| Wang G, Yu Y, Wang YZ, et al. *Role of SCFAs in gut microbiome and glycolysis for colorectal cancer therapy*. J Cell Physiol. 2019. | doi:https://doi.org/10.1002/jcp.28436 |
| Gao R, Gao Z, Huang L, et al. *Gut microbiota and colorectal cancer*. Eur J Clin Microbiol Infect Dis. 2017;36(5):757–769. | doi:https://doi.org/10.1007/s10096-016-2881-8 |
| Manzat-Saplacan RM, Mircea PA, Balacescu L, et al. *Can we change our microbiome to prevent colorectal cancer development?*. Acta Oncol. 2015;54(8):1085-95. | doi:https://doi.org/10.3109/0284186X.2015.1054949 |
| Wong SH, Kwong TNY, Wu CY, et al. *Clinical applications of gut microbiota in cancer biology*. Semin Cancer Biol. 2019;55:28-36. | doi:https://doi.org/10.1016/j.semcancer.2018.05.003 |
| Wang X, Yang Y, Huycke MM. *Microbiome-driven carcinogenesis in colorectal cancer: Models and mechanisms*. Free Radic Biol Med. 2017;105:3-15. | doi: https://doi.org/10.1016/j.freeradbiomed.2016.10.504 |
| Belizário JE, Faintuch J, Garay-Malpartida M. *Gut Microbiome Dysbiosis and Immunometabolism: New Frontiers for Treatment of Metabolic Diseases*. Mediators Inflamm. 2018;2018:2037838. | doi:https://doi.org/10.1155/2018/2037838 |
| Mandal P. *Molecular mechanistic pathway of colorectal carcinogenesis associated with intestinal microbiota*. Anaerobe. 2018;49:63-70. | doi:https://doi.org/10.1016/j.anaerobe.2017.12.008 |
| Routy B, Gopalakrishnan V, Daillère R, et al. *The gut microbiota influences anticancer immunosurveillance and general health*. Nat Rev Clin Oncol. 2018;15 (6):382-396. | doi:https://doi.org/10.1038/s41571-018-0006-2 |
| Yi M, Yu S, Qin S, et al. *Gut microbiome modulates efficacy of immune checkpoint inhibitors*. J Hematol Oncol. 2018;11(1):47. | doi:https://doi.org/10.1186/s13045-018-0592-6 |
| Nelson MH, Diven MA, Huff LW, Paulos CM. *Harnessing the Microbiome to Enhance Cancer Immunotherapy*. J Immunol Res. 2015;2015:368736. | doi:https://doi.org/10.1155/2015/368736 |
| Quince C, Walker AW, Simpson JT, et al. *Shotgun metagenomics, from sampling to analysis*. Nat Biotechnol. 2017;35(9):833-844. | doi:https://doi.org/10.1038/nbt.3935 |
| Walker AW, Duncan SH, Louis P, et al. *Phylogeny, culturing, and metagenomics of the human gut microbiota*. Trends Microbiol. 2014;22(5):267-74. | doi:https://doi.org/10.1016/j.tim.2014.03.001 |

**Box 5.4 Recommended Articles of Particular Interest for Non-English-Speaking Readers**

| | |
|---|---|
| Bruneau A et al. *Le microbiote intestinal: quels impacts sur la carcinogenèse et le traitement du cancer colorectal?*. Bull Cancer. 2018;105(1):70-80. | https://doi.org/10.1016/j.bulcan.2017.10.025 |
| Landman C and Quévrain E. *Le microbiote intestinal : description, rôle et implication physiopathologique*. Rev Med Interne. 2016;37(6):418-23. | https://doi.org/10.1016/j.revmed.2015.12.012 |
| Del Campo-Moreno R et al. *Microbiota en la salud humana: técnicas de caracterización y transferencia*. Enferm Infecc Microbiol Clin. 2018;36(4):241-245. | https://doi.org/10.1016/j.eimc.2017.02.007 |
| Icaza-Chávez ME. *Microbiota intestinal en la salud y la enfermedad*. Rev Gastroenterol Mex. 2013;78(4):240-8. | https://doi.org/10.1016/j.rgmx.2013.04.004 |
| Stallmach A, Steube A. *Gastrointestinale Mikrobiota und Tumorerkrankungen – ein "pas de deux"?*. Dtsch Med Wochenschr. 2017;142(4):254-260. | https://doi.org/10.1055/s-0042-121019 |
| Steinhagen PR, Baumgart DC. *Grundlagen des Mikrobioms*. Internist (Berl). 2017;58(5):429-434. | https://doi.org/10.1007/s00108-017-0224-1 |
| Shao DT, Wei WW. 上消化道癌及其癌前病变微生物菌群研究进展. Zhonghua Liu Xing Bing Xue Za Zhi. 2018;39(3):382-386. | https://doi.org/10.3760/cma.j.issn.0254-6450.2018.03.025 |
| Ye DD et al. 宏基因组研究的生物信息学平台现状. Dongwuxue Yanjiu. 2012;33(6):574-85. | https://doi.org/10.3724/SP.J.1141.2012.06574 |
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# The Revolution of Liquid Biopsy and Single-Cell Sequencing in the Management of Colorectal Cancer

6

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**Abstract**

Accurate prediction of long-term prognostic outcomes in colorectal cancer (CRC) continues to be challenging. Previously and routinely used prediction tools have focused on pretreatment factors. However, there is an enthusiastic interest in liquid biopsy in cancer mainly with the potential findings from human studies impacting clinical practice. In CRC as well as other malignancies, liquid biopsy has the potential to detect, characterize, and monitor recurrence and response to therapy more effectively than the current and routine conventional approaches. Moreover, with technologies advancing rapidly, liquid biopsy may find a central place as a minimally invasive method in oncology research and practice. Its ability to accurately examine tumor-derived materials with high specificity and sensitivity as compared to tissue and circulating tumor markers is another reason for promising use in the clinic in the near future. Remarkably, sequencing one tumor cell at a time was achieved recently because of single-cell genomic and transcriptomic sequencing technology that has the potential to decipher cancer heterogeneity and resistance to treatments. In this chapter, the clinical impact of

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liquid biopsy, especially circulating tumor DNA (ctDNA), and single-cell sequencing in CRC are discussed based on recent studies.

**Keywords**

Liquid biopsy · Circulating tumor DNA · Next-generation sequencing · Single-cell sequencing · Biomarkers · Colorectal cancer

## 6.1 Introduction

The optimization of therapeutic guidelines for patients with CRC is still based on clinicopathological patterns, which are imperfect in predicting outcomes such as the risk of recurrence after surgery. Interestingly, real-time and accurate monitoring of cancer relapse and treatments was recently achieved because of the emerging liquid biopsy based on the detection and characterization of ctDNA, circulating tumor cells, circulating microRNAs, and exosomes (Heitzer et al. 2019; Pantel and Alix-Panabières 2019; Conway et al. 2019). Genotyping ctDNA using next-generation sequencing (NGS) technology is a modern approach to identify CRC patients at high risk of recurrence and resistance to chemotherapy. To this end, various and several omics equipment were developed to be applied to tumor materials in order to find predictive biomarkers with improved accuracy parameters for better patients' selection (Keller and Pantel 2019). In CRC, findings from early studies using liquid biopsy have created enthusiastic movement toward better patients' care in this highly heterogeneous disease. Moreover, single-cell sequencing is also transforming our understanding of disease cellular architecture in various medical fields including developmental biology (de Soysa et al. 2019) and oncology (Hovestadt et al. 2019; Winterhoff et al. 2017). This rapidly evolving field is also attracting more attention in other novel topics such as T cells profiling to monitor immune-checkpoint inhibitors (An and Varadarajan 2018). The potential impact of single-cell sequencing is mainly presented by its precision in understanding tumor behavior, clonal evolution, and heterogeneity. These features are of high interest in predicting outcomes and mechanisms of resistance to the current treatments of CRC.

## 6.2 Next-Generation Sequencing of Colorectal Cancer in the Era of Liquid Biopsy

With the arrival of advanced techniques for isolation and molecular phenotyping of circulating tumor cells (CTCs) and other tumor components such as the CellSearch® (Veridex, LLC, Raritan, NJ, USA) and the CellCollector® (GILUPI GmbH, Hermannswerder 20a, 14473, Potsdam, Germany), liquid biopsy especially ctDNA is now widely used for a reliable tracking of tumor evolution, early detection, monitoring of drug response, and diagnosis of minimal residual disease (Tie et al. 2016; Bettegowda et al. 2014; Tadimety et al. 2018; El Bairi et al. 2018; Shen et al. 2017; Andree et al. 2016; Ferreira et al. 2016) (Fig. 6.1). CRC is one of the most

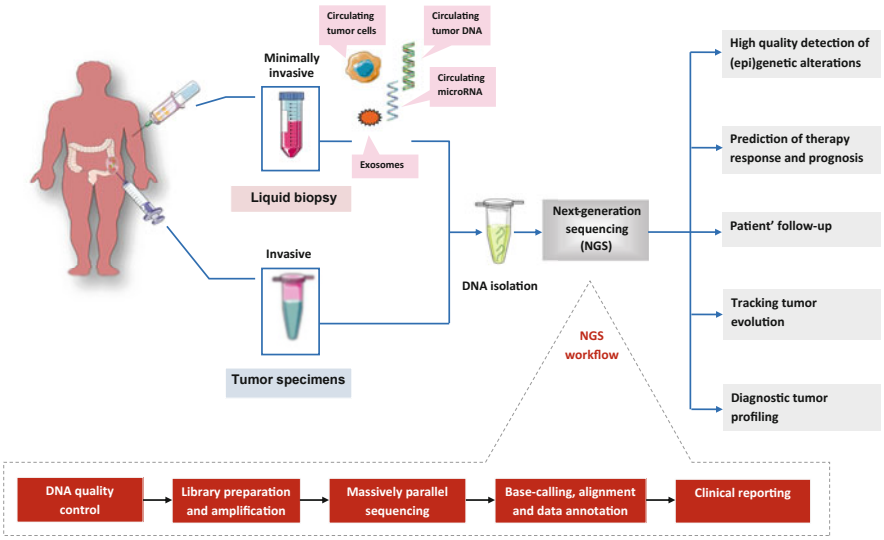


**Fig. 6.1** Global overview of NGS workflow in liquid biopsy. For comments, see text. *DNA* deoxyribonucleic acid, *NGS* next-generation sequencing

common tumors that benefited from liquid biopsy advances which support its possible future use in routine clinical care (Lim et al. 2014; Gazzaniga et al. 2015; Bachet et al. 2018; Lopez et al. 2018). CtDNA derived from both primary and metastatic cancer clones is considered as a potent biomarker for tumor progression and acquired resistance (Diaz et al. 2012; reviewed by: Khakoo et al. 2018; Gazzaniga et al. 2015 and Oellerich et al. 2017) and possesses strong diagnostic and prognostic information as demonstrated by two recent meta-analyses (Spindler et al. 2017; Tang et al. 2017). Monitoring of targeted CRC therapy needs serial invasive tissue sampling which is difficult and not feasible. In addition, tumor re-biopsy is limited by the fact of possible spatial selection bias related to considerable intra- and inter-tumor heterogeneity defined by scalable and random genetic, epigenetic, and transcriptomic events during cancer progression of different cellular subclones. Furthermore, cancer cells adapt to the targeted agents in a time-dependent manner; for example, concordance between *KRAS* status in primary tumors and metastases is still controversial. All these data together confirm that ctDNA appears as a surrogate for a real-time follow-up of treated CRC patients for better and tangible clinical benefits of these high-cost treatments.

Released CTCs and ctDNA into body fluids (passively or actively using nucleosomes) are being characterized by using strategies that require highly sensitive, robust, and precise methods such as digital PCR, "beads, emulsion, amplification, and magnetic" technology (BEAMing: combines emulsion PCR and flow cytometry to quantify sequence variants), and NGS (Diehl et al. 2006; Li et al. 2006; Hudecova 2015). Here, we focus only on studies that utilized NGS technologies holding significant improvements in terms of sensitivity as well as their great power to cover large spectrum of actionable DNA regions and multiple

target gene sequencing. Initial proof-of-concept reports provided promising data regarding the feasibility of NGS platforms combined with liquid biopsy to sequence circulating biomarkers. Based on Illumina HiSeq platform, Leary et al. used a whole-genome sequencing approach to detect genetic alterations in ctDNA of 10 late-stage CRC and breast cancer patients compared to 10 healthy subjects and identified amplification of known driver oncogenes (*CDK6* and *ERBB2*) (Leary et al. 2012). Later, Heitzer et al. used NGS of 68 CRC-associated genes using Illumina MiSeq after CTC detection in 37 stage IV CRC patients using the FDA-validated CellSearch® system (Heitzer et al. 2013). Single-cell sequencing was then applied for derived CTCs from two patients and showed mutation patterns including *APC*, *KRAS*, and *PIK3CA* in corresponding CTCs as observed in the primary tumor tissues (Heitzer et al. 2013). Braig et al. sequenced ctDNA by Illumina MiSeq to study acquired resistance to EGFR blockade in a cohort of 27 CRC patients (Braig et al. 2015). A novel EGFR (exon 12) mutation by epitope-changing mechanism was found in patients treated with panitumumab, whereas about one-third of the cases had acquired resistance by mutated *RAS* genes which may facilitate their detection using ctDNA approach (Braig et al. 2015). Significantly, a multicenter and rigorous study used Illumina MiSeq and NextSeq 500 to analyze 226 selected genes in ctDNA of histologically confirmed metastatic CRC patients with primary or acquired resistance to panitumumab and cetuximab (Siravegna et al. 2015). These NGS platforms identified novel molecular alterations including amplified *FLT3* (oncogene coding for a tyrosine kinase receptor) and mutated *MAP2K1* (oncogene coding for MEK) as potential biomarkers of therapy resistance in *RAS* wild-type CRC patients (Siravegna et al. 2015). In addition, *APC* and *TP53* mutations were detected in ctDNA that were not present in the germline DNA (Siravegna et al. 2015). Interestingly, a decline of mutated *KRAS* clones was observed when anti-EGFR therapy was withdrawn which indicates a dynamic adaptation during tumor evolution (Siravegna et al. 2015). Molecular analysis of acquired resistant CRC cells confirmed these findings and shows new sensitivity to EGFR blockade which opens the door for rechallenge therapy (Siravegna et al. 2015). Recently, Illumina MiSeq was also used to track the heterogeneity of colorectal and pancreatic cancers by sequencing a panel of 56 cancer-associated genes during metastatic progression compared to primary tumors (Vietsch et al. 2017). Importantly, 3–5 new mutations that were absent earlier were detected in ctDNA, thus providing new insights into the dynamic tumor evolution of CRC (Vietsch et al. 2017). Moreover, another study using Illumina HiSeq 2000 added evidence of using ctDNA, this time for monitoring curative surgery by detecting specific structural variants with novel insights into the possibility of follow-up by this strategy for successful CRC resection (Reinert et al. 2016). More recent evidence came from a prospective study investigating the use of ctDNA for predicting antiangiogenic response (Yamauchi et al. 2018). Tumor tissues and ctDNA from plasma samples from 21 enrolled CRC patients, who were assigned to bevacizumab, were used for sequencing a panel of 90 cancer-associated genes using Illumina HiSeq 2500 platform. A decrease of mutant allele frequency was significantly noticed at remission with a remarkable increase after progression ($p < 0.001$) (Yamauchi et al. 2018). Novel predictive mutations for VEGF blockade in *CREBBP* and *FBXW7* oncogenes were detected by this platform

suggesting a crucial role of NGS in providing new data related to altered genes during CRC progression based on ctDNA (Yamauchi et al. 2018).

In the reports with a relatively large sample size, the combination of NGS technology and ctDNA approach provided a unique opportunity to explore additional therapeutic strategies in CRC. In this perspective, Bachet et al. compared ctDNA and tissue-based analyses of *RAS* mutations in a prospective multicenter trial (NCT02502656) enrolling metastatic CRC ($n = 412$) in order to test the concordance between these two approaches (Bachet et al. 2018). Colon Lung Cancer V2 AmpliSeq panel was used for NGS analyses of tumor RAS status on bulk tumors and ctDNA (Bachet et al. 2018). An excellent concordance between the two strategies was noticed. Therefore, these findings may impact the future randomized and controlled trials enrolling metastatic CRC for anti-EGFR-targeted treatments and requiring stratification by *RAS* status. Moreover, this may also reduce the time of mutational status determination mainly based on bulk tumor samples. Another study ($n = 261$, ASPECCT) aimed to assess the ctDNA analysis for *RAS* status as a minimally invasive method in patients with metastatic CRC treated with the anti-EGFR monoclonal antibody panitumumab (Peeters et al. 2019). At baseline, the group of patients with a highly frequent mutated *RAS* had poor prognostic outcomes (Peeters et al. 2019). Notably, rechallenge with anti-EGFR therapy after acquired resistance seems to be feasible in a category of metastatic CRC based on ctDNA sequencing (Cremolini et al. 2019). This was demonstrated in a multicenter single-arm phase II trial (NCT02296203) that investigated cetuximab every 2 weeks combined with irinotecan in patients who were previously resistant to this regimen (Cremolini et al. 2019). In fact, the use of ctDNA for *RAS* status determination in this population was supportive of patients' selection (Cremolini et al. 2019). As expected, patients with wild-type status had significantly improved progression-free survival (HR: 0.44; 95% CI: 0.18–0.98; $p = 0.03$) (Cremolini et al. 2019).

In the Australian multicenter cohort ($n = 96$) that enrolled CRC patients with stage III, ctDNA was quantified in postsurgical plasma samples and correlated with recurrence-free survival (RFS) (Tie et al. 2019). The presence of ctDNA was associated with a significantly reduced RFS (HR: 3.8; 95% CI: 2.4–21.0; $p < 0.001$ (Tie et al. 2019). When adjusting for confounding clinicopathological variables, postsurgical ctDNA status significantly predicted RFI (HR: 7.5; 95% CI: 3.5–16.1; $p < 0.001$) (Tie et al. 2019). This means that the use of ctDNA in stage III CRC treated with adjuvant chemotherapy after surgery may represent an important opportunity to define a subgroup of patients that require additional therapies (Tie et al. 2019). Thus, the ability of this monitoring approach in predicting the high risk of early relapse. Similarly, Reinert et al. investigated this strategy for recurrence detection and patients' stratification using a multicenter and prospective cohort ($n = 130$) of stages I–III CRC (Reinert et al. 2019). A total of 829 plasma samples were collected before and after surgical resection as well as during longitudinal surveillance (Reinert et al. 2019). During follow-up, risk of disease recurrence seems to be experienced more than 40 times in patients with positive ctDNA as compared to those with negative status (HR: 43.5; 95% CI: 9.8–193.5, $p < 0.001$) (Reinert et al. 2019). Importantly, ctDNA status was found as an independent

predictor of relapse on multivariate analysis (Reinert et al. 2019). Moreover, ctDNA was detected in recurrent patients up to 16.5 months before the radiological examination during follow-up (Reinert et al. 2019). Promisingly, ctDNA also provided information on actionable mutations in 81.8% of the samples of patients who experienced relapse (Reinert et al. 2019). These findings were confirmed in a recent study ($n = 58$) that evaluated the impact of serial monitoring based on ctDNA (319 samples) as compared to the conventional standard modalities in nonmetastatic CRC (Wang et al. 2019). The authors demonstrated that ctDNA positivity preceded the evidence of recurrence using the radiographic imaging by a median of 3 months (Wang et al. 2019). Recently, a large study enrolled 801 CRC patients and 1021 normal controls and used ctDNA methylation markers to develop a prediction model for diagnostic and prognostic purposes (Luo et al. 2020). This model reached an area under curve of 0.96, which is suggestive of a high accuracy in categorizing CRC patients from normal subjects (Luo et al. 2020). Moreover, the model was also able to predict survival outcomes in CRC ($p < 0.001$) (Luo et al. 2020). For surgery, ctDNA showed also an advantage in predicting survival after liver resection in metastatic CRC (Narayan et al. 2019). Detection of peripheral ctDNA with mutated *TP53* was associated with a significantly reduced disease-specific survival ($p = 0.024$) (Narayan et al. 2019).

The emerging companion diagnostic tests using NGS such as Illumina HiSeq-based Guardant 360™ (Lanman et al. 2015) developed by Guardant Health® (Redwood City, CA, USA) are becoming widely used to detect genomic alterations in ctDNA. This targeted NGS assay represents an encouraging strategy for genomic profiling of patients with CRC in clinical practice (Strickler et al. 2017; Schwaederle et al. 2017; Zill et al. 2017). However, ctDNA fragments in the blood of cancer patients are mainly released by tumors cells after apoptosis. This fact may limit the accuracy of ctDNA to evaluate surviving tumor clones that may effectively predict resistance to treatments.

NGS combined with liquid biopsy requires expertise and importantly budget, but it will be more affordable with the remarkable decrease of costs of these emerging sequencing technologies. Notably, this dynamic molecular analysis mainly based on ctDNA seems to be more sensitive for tracking the clonal evolution of tumor cells in CRC as compared to the standard tumor tissue biopsy (Siena et al. 2018). Studies comparing the available NGS platforms adapted to liquid biopsy are needed to confirm these important findings. Promisingly, various clinical trials enrolling CRC patients (see Table 6.2) are using ctDNA for various purposes including monitoring therapy and more insights on this hot topic will appear in the future. The current international guidelines do not recommend the use of ctDNA yet in the management of CRC.

## 6.3 Emergence of Single-Cell Sequencing to Unravel Tumor Heterogeneity in Colorectal Cancer

Cellular heterogeneity in tumors is one of the most stochastic events that limit the effects of our current weapons against cancer. Genetic intra-tumor heterogeneity is an expected consequence of defective DNA replication. Sequencing DNA from biopsy and surgical specimens using current NGS technologies does not represent the whole picture of tumor molecular alterations. Tumor mutations increase dynamically with time allowing tumor subclones to acquire many needed properties such as survival and resistance to treatments (Lee and Swanton 2012; Burrell et al. 2013). Tracing tumor evolution and heterogeneity has generated new insights into this complex process and is now possible at a cancer-cell level in bulk tissue and liquid biopsy approaches with the newly developed "single-cell sequencing" methods (Macaulay and Voet 2014; Zhang et al. 2016; Ellsworth et al. 2017) (Fig. 6.2). Importantly, single-cell sequencing has the potential to map cancer genome aberrations cell by cell and therefore to identify rare resistant tumor cells to adjuvant therapies leading to tumor recurrence as well as cells of the tumor microenvironment which may be considered as personalized biomarkers (Navin and Hicks 2011). Furthermore, dynamic epigenomic and transcriptomic tumor heterogeneity may also be studied by single-cell sequencing to illuminate these phenomena (Macaulay and Voet 2014; Roerink et al. 2018). The beginning of this important advance was inaugurated by Nicholas Navin (PhD) (currently at MD Anderson Cancer Center) who developed various methods for this new field.

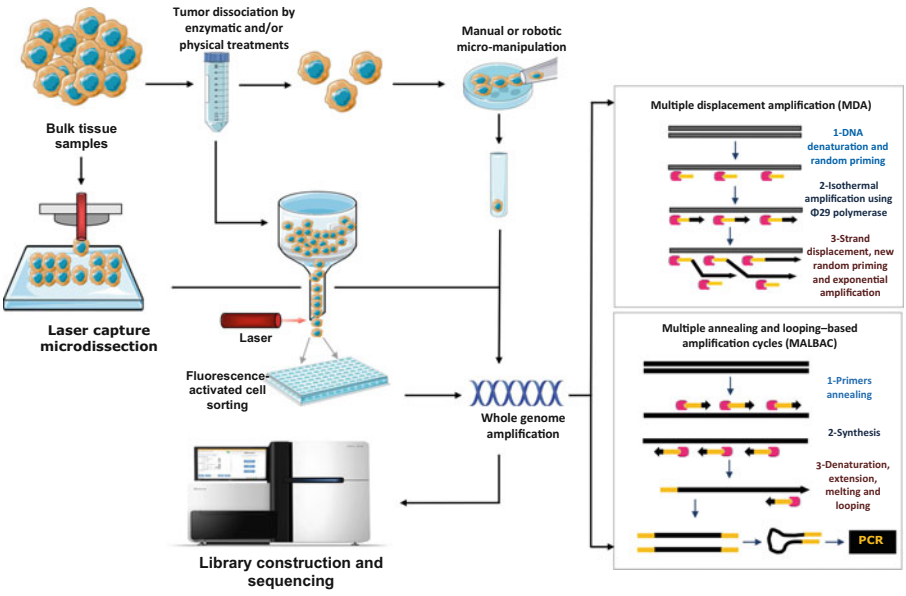


**Fig. 6.2** Simplified workflow of single-cell isolation and sequencing. For comments, see text

Practically, first, single cells are isolated from dissociated tumor tissues using well-established protocols and methods such as flow-assisted cell sorting (a flow cytometry-based technique), laser capture microdissection, and micromanipulation methods (Navin 2014). Then, this step is followed by whole-genome amplification that utilizes various methods such as multiple displacement amplification (MDA) or multiple annealing and looping-based amplification cycles (MALBAC) (Zhang et al. 2016). MDA is simple and enables a rapid DNA amplification with large weight products at constant temperature using high proofreading enzyme preferentially Phi29 (Φ29) polymerase (Navin and Hicks 2011; Lage et al. 2003). In contrast, MALBAC is PCR dependent and requires picograms of DNA templates, and it was found ideal for single-cell sequencing as it allows high genome coverage and isothermal linear amplification based on several phases encompassing (1) melting, (2) random priming, (3) amplification extension, and (4) looping of the isothermal amplicons to inhibit further exponential amplification in order to minimize biases followed by a final PCR step (Zong et al. 2012) (reviewed by Gawad et al. 2016). Once these steps are complete, library construction, NGS, and bioinformatic analysis are performed to identify copy-number variations, structural changes, mutations, and other epigenetic and transcriptomic aberrations. Earlier, Zong et al. performed single-cell sequencing of genetic alterations in a CRC cell line (SW480) using MALBAC-based amplification along with Illumina HiSeq 2000 and Illumina MiSeq sequencing platforms and were able to achieve 93% of genome coverage and identified single-nucleotide and copy-number variations in a single tumor cell (Zong et al. 2012). From a clinical standpoint (Table 6.1), Yu et al. applied MDA-single-cell sequencing to a CRC case and identified *APC* and *TP53* oncogene mutations as early events in a major tumor subpopulation and mutated *CDC27* (cell division cycle 27) and *PABPC1* (poly(A) binding protein cytoplasmic 1) in the minor clone (Yu et al. 2014). The authors claimed that because of the absence of mutated *APC* and *TP53* in the minor clone, CRC may have a biclonal cell origin (Yu et al. 2014). Contrary to the previous findings, Wu et al. employed single-cell exome sequencing on two CRC patients and observed that both adenomatous polyps and cancer are of monoclonal origin and have in common some similar mutations in the same pathway (Wu et al. 2017). Distinct CRC subclones that accumulated diverse nonrandom mutation profiles in FGFR, PI3K-Akt, and GPCR (G protein-coupled receptor) pathways were also noted, suggesting that CRC diversify into various different sub-tumors (Wu et al. 2017). Another recent report from Navin's team traced the evolution of CRC clonality between primary and metastatic tumors in two patients using MDA and degenerate oligonucleotide-primed (DOP)-PCR-based single-cell nucleus sequencing for mutational and copy-number profiling, respectively (Leung et al. 2017). After preparation of nuclear suspensions from frozen CRC tissues and their deposit into individual wells for whole-genome amplification, sequencing with Illumina HiSeq 2000/4000 found that dissemination of cancer cells in the two patients required known driver mutations such as *TP53*, *APC*, *NRAS*, *KRAS*, and cyclin-dependent kinase 4 (*CDK4*) during an extended period of time supporting therefore the late-dissemination model of metastasis (Leung et al. 2017). This study supports the notion that despite in advanced stages,

**Table 6.1** Summary of studies that used single-cell sequencing to study colorectal cancer heterogeneity

| Author/year | Sample size, specimen, and setting | NGS platform | Key findings |
|---|---|---|---|
| Zhang et al. (2020) | • $N = 1$<br>• Colorectal cancer (CRC) liver metastases | Illumina NextSeq 500 (RNA seq) | • Expression of 93 cell cluster deregulated genes in tumor-infiltrating cells is correlated with patients' survival outcomes<br>• IL-17 signaling pathway was enriched in granulocytes of CRC metastases<br>• Granulocytes of CRC liver metastases had activated Wnt signaling pathway |
| Li et al. (2019) | • Six patients with familial adenomatous polyposis (FAP) and one patient with MUTYH-associated polyposis | Illumina HiSeq 4000 | • Carcinogenic events in FAP may happen long before clinically detectable adenomas<br>• Transcriptomic sequencing showed that normal epithelium of patients with FAP has enhanced metabolic and proliferative processes<br>• Reprogramming of metabolism of carbohydrates occurs in precancerous adenomas |
| de Vries et al. (2019) | • $N = 35$<br>• Primary CRC tissues with matched lymph nodes ($n = 26$), healthy mucosa ($n = 17$), and peripheral blood ($n = 19$)<br>• Mainly stages I–III | Illumina HiSeq 4000 (RNA seq) | • Single-cell sequencing evidenced an enrichment of colorectal tumors by a subpopulation of lymphocytes with $CD103^+$/$CD69^+$ markers that were previously undervalued, exhibiting cytotoxicity, and was the most abundant in CRC with MMR deficiency<br>• Presence of these immune cells correlated with γδ T cells, which were notably present in tumors with MMR deficiency |
| Bolhaqueiro et al. (2019) | • Patient-derived organoids from 11 CRC patients and frozen tissues | Illumina NextSeq 500 | • Heterogeneity of copy-number alterations (CNAs) in patient-derived organoids was revealed by single-cell karyotype sequencing<br>• Novel karyotypes were evolved over time by monoclonal cell lines |

(continued)

**Table 6.1** (continued)

| Author/year | Sample size, specimen, and setting | NGS platform | Key findings |
|---|---|---|---|
| Zhang et al. (2018, 2019) | • $N = 12$<br>• Tumors, adjacent tissues, and peripheral blood<br>• Various stages of CRC | Illumina HiSeq 4000 (RNA seq) | • Single-cell transcriptomic analysis and TCR tracking of T cells from tumors, adjacent tissues, and peripheral blood provided 11,138 single transcriptomes including 20 categorized T-cell subpopulations with different functions and clonalities<br>• CRC patients with microsatellite-instable tumors had enriched microenvironment by $CXCL13^{+}BHLHE40^{+}$ $T_H1$-like cells<br>Data of this study are publically available at: http://crctcell.cancer-pku.cn/ |
| Roerink et al. (2018) | • Patient-derived organoids from three treatment-naïve CRC patients | Illumina X10 and Illumina HiSeq 2000 (RNA seq) | • Extensive somatic mutational diversification in CRC cells was observed as compared to normal cells<br>• Acquisition of most of mutations evolved throughout the final dominant tumor clonal expansion<br>• Phylogenetically and closely related cells of the same tumor had distinct response to anticancer treatments |
| Bian et al. (2018) | • $N = 10$<br>• Primary colorectal tumors and lymphatic or distant metastases | Illumina HiSeq 4000 | • The CRC methylome can significantly differ between tumor clones |
| Marie et al. (2018) | • CRC-derived cell lines and fresh colorectal tumor samples | Illumina HiSeq | • Single-cell sequencing with high-quality whole-genome profiling is achievable using an inexpensive scalable developed instrument |
| Liu et al. (2018) | • $N = 2$<br>• Primary colon tumors | Illumina MiSeq and Illumina HiSeq | • Single-cell sequencing of colonic cancer stem cells indicated that every patient had particular copy-number alterations<br>• Copy-number profiles in cancer stem cells and differentiated tumor cells were similar with some |

(continued)

**Table 6.1** (continued)

| Author/year | Sample size, specimen, and setting | NGS platform | Key findings |
|---|---|---|---|
| | | | regional differences suggesting that these alterations occurred at an early stage of colon carcinogenesis |
| Leung et al. (2017) | • $N = 2$<br>• Frozen primary CRC tissues and matched liver metastases<br>• Microsatellite-stable, invasive, and stage IV CRC | Illumina HiSeq 2000 and Illumina HiSeq 4000 | • Monocloncal and biclonal seeding mediated liver metastasis in CRC after accumulating a large number of mutations<br>• Single-cell sequencing supports the model of late dissemination of CRC metastasis |
| Wu et al. (2017) | • $N = 2$<br>• CRC | Illumina HiSeq 4000 | • Colorectal adenoma and CRC have monoclonal origin<br>• CRC develops into different tumor subclones with heterogeneous mutational features (GPCR, PI3K-Akt, and FGFR pathways) |
| Yu et al. (2014) | • $N = 1$<br>• Colon adenocarcinoma (T3N0M0) | Illumina (not specified) | • Single-cell sequencing found two independent clones in tumor cell populations with distinct mutational profiles<br>• Mutated *APC* and *TP53* genes were characteristic of early oncogenic events in the major clone<br>• The minor clone had predominant mutated *CDC27* and *PABPC1* and absent mutated *TP53* and *APC* |

*Akt* protein kinase B, *APC* adenomatous polyposis coli, *BHLHE40* class E basic helix-loop-helix protein 40, *CDC27* cell division cycle protein 27 homolog, *CNAs* copy-number alterations, *CRC* colorectal cancer, *CXCL13* chemokine (C-X-C motif) ligand 13, *CD103* cluster of differentiation 103, *CD69* cluster of differentiation 69, *FGFR* fibroblast growth factor receptor, *GPCR* G protein-coupled receptor, *MMR* mismatch repair, *PABPC1* polyadenylate-binding protein 1, *PI3K* phosphoinositide 3-kinase, *RNA* ribonucleic acid, $T_H1$ T helper 1, *TP53* tumor protein 53

CRC patients at a localized stage may be cured by surgical removal and treatment to prevent metastatic dissemination (Leung et al. 2017).

The application of single-cell sequencing on patient-derived organoids has been also investigated in CRC (Roerink et al. 2018). Intra-tumor diversification of somatic mutations in CRC was noticed compared to normal cells particularly during the final dominant expansion of the tumor clones (Roerink et al. 2018). It seems that the acquisition of the majority of mutations in CRC occurs at some stages of the final

clonal expansion (Roerink et al. 2018). Remarkably, this study showed that tumors cells with similar phylogenetic characteristics had a distinctive response to anticancer drugs (Roerink et al. 2018). Similarly, this significant clonal expansion was also detected in CRC methylome of primary tumors, lymphatic, and distant metastases (Bian et al. 2018). This suggests that epigenetic events in CRC may also undergo this phenomenon. Furthermore, sequencing of stem cells from primary colorectal tumors showed that each patient has distinct copy-number alterations (Liu et al. 2018). This genetic profile was found to be comparable to that of differentiated tumor cells with only some regional variations (Liu et al. 2018). Thus, indicating that the occurrence of these alterations may arise earlier during colon tumorigenesis (Liu et al. 2018).

In familial adenomatous polyposis (FAP) and MUTYH-associated polyposis, Li et al. demonstrated that patients had carcinogenic events before detectable tumors (Li et al. 2019). Their normal epithelium showed enhanced proliferative activities and metabolisms of peptides, nucleotides, carbohydrates, lipids, and amino acids (Li et al. 2019). Importantly, this metabolic signature revealed by single-cell sequencing seems to occur earlier in precancerous lesions (Li et al. 2019) and, therefore, a potential perspective for preventive approaches and early diagnosis. Of note, metabolic reprogramming is a hallmark of cancer (reviewed elsewhere: Hagland et al. 2013; Pavlova and Thompson 2016). Single-cell sequencing demonstrated advantages in studying the associated immune components of the tumor microenvironment in CRC. A recent report ($n = 35$) by De Vries et al. found that colorectal tumors were enriched by CD103+/CD69+ T cells which were previously underevaluated (de Vries et al. 2019). This subset of lymphocytes was highly abundant in MMR-deficient tumors and displayed notable cytotoxic properties (de Vries et al. 2019). Based on transcriptomic sequencing of 11,138 single T cells of colorectal tumors, adjacent normal tissues, and peripheral blood from 12 CRC patients, Zhang et al. categorized 20 T-cell subpopulations with different functions and clonalities (Zhang et al. 2018). Notably, patients with microsatellite-instable tumors had an enriched microenvironment by CXCL13 + BHLHE40+ TH1-like cells (Zhang et al. 2018). This lineage tracking may therefore explain the dramatic clinical improvement in terms of response to immune-checkpoint inhibitors in CRC patients with microsatellite-instable tumors (Zhang et al. 2018, 2019; Sahin et al. 2019). Single-cell RNA sequencing was also applied to study the immune contexture of metastases of CRC and showed that associated granulocytes had enriched IL-17 and activated Wnt signaling (Zhang et al. 2020). These two mechanisms have a significant role in evading cancer immunosurveillance (Zhang et al. 2020). Therefore, this method unraveled actionable information for modulating immune microenvironment of liver metastases to boost patients' outcomes (for review, see: Wang et al. 2018; Galluzzi et al. 2019).

In addition to its benefits in mapping the heterogeneity of colorectal tumors cells, the place of single-cell sequencing in oncology has also expanded to the study of the immune landscape of CRC particularly with emergence of immune-checkpoint blockade. Importantly, the arrival of recently developed inexpensive and scalable instruments for single-cell sequencing (Marie et al. 2018) may provide additional information on this hot topic and therefore illuminate CRC genetics.

## 6.4 Future Perspectives

The role of detecting, counting, and characterizing the molecular biology of circulating biomarkers, CTCs, and cellular fractions like ctDNA has been increasingly characterized in colorectal neoplasms, along with the development observed in other tumor types. Clinical applications of liquid biopsy are diverse, developed to tackle relevant unmet needs for cancer patients. Accordingly, clinical research is investigating multiple applications for the diagnosis of early-stage CRC, as a prognostic marker in early-stage disease and as a monitoring tool in metastatic patients. The study of CTCs has been suggested for early and advanced disease to inform on the prognosis and enhance the formulation of risk-adapted approaches. Additionally, the possibility to characterize tumors with no or less need of tissue is the main reason emphasizing a broader application, reducing the discomfort and procedural complications for patients.

Risk definition of early CRC patients is a promising application of liquid biopsy. For instance, in patients with rectal cancer, the extramural invasion of veins is associated with a higher risk of relapse, and its identification plays a critical role in the selection of multimodal therapies. In this context (Table 6.2), the role of CTCs has been suggested and is under investigation, to assess whether CTCs in early rectal cancer can recapitulate malignant venous involvement (NCT02579278). In the broader context, the concept of postsurgical residual disease is explored across several histology types (NCT03189576). Interestingly, studies are assessing the reproducibility and clinical performance of CTC assays on blood and other biologic fluids, like urine (NCT02838836). Applications in next-generation sequencing are largely investigated (NCT03312374). Support in refining the clinical detection of CRC in the diagnostic phase is also conceptualized, in conjunction with endoscopy (NCT02665299) or alone as a strategy for early detection (NCT02578264). Some patients with advanced disease or liver-predominant relapsed cancer at stage IV can be eligible for curative treatments, mainly locoregional resections or ablations followed by systemic treatments. In this setting, the counting and assessment of CTCs has been proposed to help identify the patients more likely to have a significant benefit from the more aggressive strategy and reduce the exposure to patients deriving no benefit, including a reduction of possible harm (NCT03295591). In disease monitoring of patients at higher risk or resected stage IV patients with no evidence of disease, after the completion of therapies, the role of circulating biomarkers is explored. However, the availability of a biomarker able to support an earlier diagnosis of relapses and improve outcome is missing and the prognostic role of an earlier diagnosis is investigational. When disease has spread in distant sites, the detection and study of CTCs can inform on treatment decision, especially in settings where a primary resistance to standard therapies is observed. The possibility to study and track the changes in molecular profiles of CRC under therapies can offer the possibility to study the primary and identify the acquired mechanisms of resistance, including emerging genomic alterations. In early disease, when cancer is resected and adjuvant treatments delivered, CTCs can be embedded in a multimodal approach of monitoring, as a circulating cellular biomarker of prognosis.

**Table 6.2** Selected clinical trials based on liquid biopsy applications for colorectal cancer management

| Clinical trial identifier | *N* | Status | Purpose | Study completion date[a] | Sponsor |
|---|---|---|---|---|---|
| NCT03637686 (IMPROVE trial) | 1800 | Recruiting | Investigation of ctDNA as a biomarker of subclinical residual disease and risk of recurrence in CRC | June 2025 | University of Aarhus in collaboration with Aarhus University Hospital, Denmark |
| NCT04050345 (TRACC trial) | 1000 | Recruiting | Assessment of ctDNA to detect minimal residual disease and relapse in CRC earlier than conventional methods | December 2024 | Royal Marsden NHS Foundation Trust, United Kingdom |
| NCT03517332 | 10,000 | Recruiting | Assessment of the feasibility of detecting ctDNA of several cancers including CRC in the peripheral blood | December 2019 | Quantgene Inc. |
| NCT03809403 (ADNCHIR) | 40 | Recruiting | Study of the influence of surgical techniques for CRC on the concentration of ctDNA and circulating tumor cells | August 2019 | Assistance Publique—Hôpitaux de Paris, France |
| NCT04120701 | 1980 | Not yet recruiting | Assessment of ctDNA as a biomarker for monitoring CRC after surgery | December 2027 | Centre Hospitalier Universitaire Dijon, France |
| NCT04089631 (CIRCULATE) | 4812 | Not yet recruiting | Comparison of disease-free survival in stage II CRC patients who are positive for postoperative ctDNA treated with or without capecitabine | July 2026 | Technische Universität Dresden, Germany |
| NCT03748680 (IMPROVE-IT trial) | 64 | Recruiting | Monitoring of molecular biological response to adjuvant chemotherapy in CRC patients based on ctDNA in a phase II trial | October 2025 | Aarhus University Hospital, Denmark |
| NCT04084249 (IMPROVE-IT2 trial) | 254 | Not yet recruiting | Use of ctDNA and imaging to guide postoperative surveillance to detect recurrent disease and stratify patients for curative interventions | December 2025 | Aarhus University Hospital, Denmark |
| NCT01198743 | 261 | Completed | Detection of mutations in *KRAS*, *NRAS*, *BRAF*, *APC*, *TP53,* and microsatellite instability genes in ctDNA | May 2017 | Assistance Publique—Hôpitaux de Paris, France |

| | | | | | |
|---|---|---|---|---|---|
| NCT03416478 | 50 | Recruiting | Evaluation of ctDNA as a predictive biomarker for tumor recurrence of stage II and III CRC | December 2020 | Sixth Affiliated Hospital, Sun Yat-sen University, China |
| NCT01212510 | 200 | Completed | Evaluation of mutated ctDNA and CTCs during treatment | July 2016 | University Hospital, Rouen, France |
| NCT03259009 | 73 | Not yet recruiting | Predictive value of mutated *RAS* in ctDNA for efficacy of anti-EGFR reintroduction treatment in metastatic CRC patients | June 2020 | Association des Gastroentérologues Oncologues, France |
| NCT03312374 | 350 | Recruiting | Exploring the correlation between mutational status of ctDNA and prognosis using NGS in CRC patients with early and intermediate stages and its utility as a clinical index for predicting postoperative relapse | March 2020 | Sun Yat-sen University, China |
| NCT02502656 | 425 | Recruiting | Evaluation of the concordance of mutated *RAS* in ctDNA and tumor tissue samples | August 2017 | Association des Gastroentérologues Oncologues, France |
| NCT02827565 | 30 | Completed | Exploring the concordance of mutated *KRAS*, *BRAF*, and *NRAS* in ctDNA using NGS and genetic profiles obtained from tissue samples | January 2017 | Institut de Cancérologie de Lorraine, France |
| NCT02872779 | 74 | Recruiting | Predictive prospective evaluation of metastatic CRC patients treated with first-line chemotherapy (5 fluorouracil +/− oxaliplatin +/− irinotecan +/− targeted therapy) using ctDNA as defined by the RECIST 1.1 guidelines | August 2020 | University Hospital, Rouen, France |
| NCT02842203 | 150 | Recruiting | Evaluation of ctDNA as a prognostic biomarker and as a monitor of disease recurrence in stage III CRC | September 2021 | University of Pittsburgh in collaboration with National Cancer Institute (NCI), USA |
| NCT03038217 | 300 | Not yet recruiting | Investigation of ctDNA value for diagnosis, therapy, and follow-up of patients with surgically resectable CRC using NGS | December 2021 | Peking Union Medical College Hospital, China |

(continued)

**Table 6.2** (continued)

| Clinical trial identifier | *N* | Status | Purpose | Study completion date[a] | Sponsor |
|---|---|---|---|---|---|
| NCT03189576 | 37 | Recruiting | Assessment of ctDNA as a noninvasive approach for residual disease monitoring in CRC patients after primary surgery | August 2021 | Tampere University Hospital, Finland |
| NCT02838836 | 110 | Recruiting | Use of ctDNA, CTCs, and disseminated tumor cells (DTCs) in blood, urine, and bone marrow of patients with resectable solid primary cancers including CRC for personalized medicine | June 2021 | University of Missouri-Columbia, USA |
| NCT02579278 | 40 | Recruiting | Exploring ctDNA to predict extramural venous invasion in rectal cancer | November 2017 | Royal Marsden NHS Foundation Trust, UK |
| NCT02665299 | 206 | Completed | Detection of CRC in subjects undergoing diagnostic colonoscopy using ctDNA | August 2017 | Pathway Genomics, UK |
| NCT02318901 | 90 | Active, not recruiting | Characterization of ctDNA changes in patients with various cancers including CRC enrolled in phase Ib/II of pembrolizumab[b] | December 2018 | Western Regional Medical Center, USA |
| NCT02997241 | 500 | Not yet recruiting | Exploring the impact of ctDNA as a predictor of recurrence and treatment decisions in colon cancer | September 2023 | MyGenostics Inc., Beijing, China |
| NCT02186236 | 84 | Completed | Detection of oncogenic mutations in the urine and blood of lung and CRC patients | September 2016 | Memorial Sloan Kettering Cancer Center, UK |
| NCT02948985 | 100 | Not yet recruiting | Analysis of RAS status on CTCs and ctDNA as a predictive biomarker for therapy response | December 2019 | Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, China |
| NCT03295591 | 77 | Recruiting | Evaluation predictive value of incorporating CTC enumeration and sequencing in metastatic CRC patients who are planning to have curative resection of liver metastases | March 2020 | The University of Hong Kong, China |

| | | | | | |
|---|---|---|---|---|---|
| NCT02556281 | 216 | Recruiting | Comparison of the prognostic value of circulating KRAS point mutations and methylated RASSF2A in ctDNA and CTCs | September 2019 | University Hospital, Rouen, France |
| NCT02751177 | 236 | Active not recruiting | Comparison of *RAS* and *BRAF* genotyping results achieved in analyzing ctDNA using OncoBEAM™ with those achieved by standard genotyping techniques | January 2018 | Institut de Cancérologie de Lorraine, France |
| NCT02578264 | 26 | Active not recruiting | Characterization ctDNA for early detection of CRC | July 2018 | Scripps Translational Science Institute in collaboration with Sequenom, Inc. USA |
| NCT03087071 | 84 | Recruiting | Use of ctDNA as a predictive biomarker (*EGFR* S492R, *KRAS,* or *NRAS* in exons 2, 3, or 4; or *BRAF* codon 600 mutations) for panitumumab in combination with trametinib in cetuximab-refractory stage IV CRC | December 2021 | M.D. Anderson Cancer Center in collaboration with Amgen and Novartis, USA |
| NCT02813928 | 473 | Recruiting | Diagnostic and prognostic value of ctDNA for CRC patients' follow-up after curative treatment | December 2020 | University Hospital, Limoges, France |
| NCT02423954 | 49 | Active not recruiting | Quantification of changes in ctDNA in enrolled patients | April 2018 | Western Regional Medical Center, USA |

*APC* adenomatous polyposis coli, *CRC* colorectal cancer, *CTCs* circulating tumor cells, *ctDNA* circulating tumor DNA, *DTCs* disseminated tumor cells, *EGFR* epidermal growth factor receptor, *KRAS* V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog, *NGS* next-generation sequencing, *NRAS* neuroblastoma ras viral oncogene homolog, *RASSF2A* Ras association domain-containing protein 2, *RECIST* response evaluation criteria in solid tumors, *TP53* tumor protein 53. Data from ClinicalTials.gov, accessed 11/10/2019; studies with unknown status were excluded

[a]Estimated (may be changed with time)

[b]As a secondary measure

*N* enrollment

Evaluation of ctDNA as a prognostic biomarker and to monitor the recurrence of disease is suggested in stage III CRC, known for a higher likelihood to relapse (NCT02842203). To date, this is not a standard approach to follow-up patients and the utilization in clinic is experimental. The principal investigation built on a liquid biopsy approach is to improve the understanding and prediction of therapeutic benefit of targeted agents and immunotherapy. The validation of the technique and its applications to assist treatment decisions has primarily been developed as a substitute of tissue biopsy or complement pathological findings. The monitoring of changes in the molecular profile of malignant cells can be explored using liquid biopsy (NCT01212510), to predict response to standard therapies, like anti-EGFR, or experimental compounds. Interestingly, a prospective validation of the change in ctDNA related to the disease response evaluated at imaging is ongoing (NCT02872779).

Applications of liquid biopsy-based techniques remain the subject of major interest for research, across several settings and clinical scenarios. As a noninvasive method for the analysis of the molecular features of CRC, the role of liquid biopsy is emphasized currently in the treatment decision and, more interestingly, in disease monitoring. In fact, the analysis of biological fluids, including and not limited to blood samples, can be promisingly ensured with noninvasive or less invasive procedures, optimizing adherence and compliance with interventions. However, the high variability of methodologies and platforms of data analysis suggest an effort of harmonization, with specifications, quality assessment protocols, and minimal requirements of performance defined, currently. In summary, ctDNA and CTCs can be used for the determination of genomic, epigenetic, and immunological alterations in CRC patients, across several indications: risk assessment, diagnosis, prognosis, treatment response, in-depth study of tumor biology, and monitoring for recurrence early detection. More importantly, liquid biopsy offers the unique possibility to provide a dynamic assay of patients, beyond the crystallized and fixed paradigm of knowledge derived from biopsies—especially when information on archival samples is used to decide treatments in pretreated patients. The exploration of ctDNA and CTCs derived information is actually rapidly evolving and can space far beyond the mere analysis of single or a set of mutations and can include the study of DNA fragment size, epigenetic modifications, and chromatin organization and nucleosome footprints. For this, research is refining the paradigm of molecular-driven development of therapeutic strategies, addressing molecular pathways and not only single genetic alterations, studied as a network, to describe a comprehensive landscape for precision medicine. Clinical trials investigating the clinical impact and utility of liquid biopsy to change CRC management are ongoing. Their findings are expected to support the implementation of liquid biopsy to deliver precision medicine in CRC (IJzerman et al. 2018). Moreover, this approach has health economic potential for serial monitoring. In fact, decision for discontinuation of inactive treatments may be supported by liquid biopsy (IJzerman et al. 2018).

## 6.5 Conclusion

Recently, attention is turning to minimally invasive liquid biopsy, which enables characterization of tumor components such as ctDNA in human body fluids mainly blood. In addition to CTCs, ctDNA profiling has been widely studied in cancer particularly CRC to accurately trace evolution of tumor genomics during progression and treatment as well. Moreover, single-cell sequencing advances have resolved the obstacle of low DNA quantity from biopsy materials and limited number of tumor cells and it will uncover more details about tumor evolution mechanisms in the next few years. However, as we begin to dissect the complex role of genetics in CRC and with the emergence of these cutting-edge advanced technologies, this "fourth-generation sequencing" progress is not perfect yet and it is still suffering from technical challenges (recommended focusing reviews and additional data on this topic can be found in Boxes 6.1 and 6.2). Findings from prospective clinical studies are required to transfer liquid biopsy and single-cell sequencing to clinical practice in the era of precision oncology.

**Box 6.1 Additional Information on the Emergence of Single-Cell Sequencing in Oncology**

| Recommended articles from the Nicholas Navin's team[a] | DOI |
|---|---|
| Casasent AK, Schalck A, Gao R, et al. *Multiclonal Invasion in Breast Tumors Identified by Topographic Single Cell Sequencing*. Cell. 2018;172(1-2):205–217.e12. | https://doi.org/10.1016/j.cell.2017.12.007 |
| Wang Y, Navin NE. *Advances and Applications of Single Cell Sequencing Technologies*. Mol Cell. 2015;58(4):598-609. | https://doi.org/10.1016/j.molcel.2015.05.005 |
| Kim C, Gao R, Sei E, et al. *Chemoresistance Evolution in Triple-Negative Breast Cancer Delineated by Single-Cell Sequencing*. Cell. 2018;173(4):879–893.e13. | https://doi.org/10.1016/j.cell.2018.03.041 |
| Navin NE. *The first five years of single-cell cancer genomics and beyond*. Genome Res. 2015;25(10):1499-1507. | https://doi.org/10.1101/gr.191098.115 |
| Navin N, Kendall J, Troge J, et al. *Tumor evolution inferred by single cell sequencing*. Nature. 2011;472(7341):90-94. | https://doi.org/10.1038/nature09807 |
| van den Bos H, Bakker B, Spierings DCJ, et al. *Single-cell sequencing to quantify genomic integrity in cancer*. Int J Biochem Cell Biol. 2018;94:146–150. | https://doi.org/10.1016/j.biocel.2017.09.016 |

(continued)

**Box 6.1** (continued)

| *Books and research protocols* | |
|---|---|
| Roth A, McPherson A, Laks E, et al. *Clonal genotype and population structure inference from single-cell tumor sequencing*. Nat Methods. 2016;13(7):573-6. | https://doi.org/10.1038/nmeth.3867 |
| Baslan T, Kendall J, Rodgers L, et al. *Genome wide copy number analysis of single cells*. Nature protocols. 2012;7(6):1024-1041. | https://doi.org/10.1038/nprot.2012.039 |
| Tseng FG, Santra TS. *Essentials of Single-Cell Analysis*. 1st ed; Springer-Verlag: Berlin/Heidelberg, Germany, 2016. | https://doi.org/10.1007/978-3-662-49118-8 |
| Xu Y, Zhou X. *Applications of Single-Cell Sequencing for Multiomics*. In: Huang T. (eds) Computational Systems Biology. Methods in Molecular Biology. Humana Press, New York, NY. 2018;1745: 327-374. | https://doi.org/10.1007/978-1-4939-7717-8_19 |
| *Helpful links and tools* | |
| The Navin laboratory at MD Anderson Cancer Center | • http://www.navinlab.com/navinlab/home.html<br>• https://www.mdanderson.org/research/departments-labs-institutes/labs/navin-laboratory.html |
| MONOVAR[b] | https://bitbucket.org/hamimzafar/monovar |
| Video articles about single-cell sequencing from JoVE[c] | https://www.jove.com/search?q=single+cell+sequencing&filter_type_1=and&filter_val_1=&filter_type_2=or&filter_val_2=&filter_type_3=not&filter_val_3=&authors=&from=&to=&exclude_sections=&exclude_series |

[a]A pioneer in single-sequencing technology (https://scholar.google.com/citations?user=e4mp7GoAAAAJ&hl=en)
[b]A tool for single-nucleotide variants detection in single-cell sequencing data
[c]Journal of Visualized Experiments

## Box 6.2 Recommended Articles from Highly Accessed *Medline*-Indexed Journals and Books

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(continued)

**Box 6.2** (continued)


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# Overview of Cost-Effectiveness and Limitations of Next-Generation Sequencing in Colorectal Cancer

7

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**Abstract**

In the last decade, the introduction of next-generation sequencing (NGS) and bioinformatic tools for medical research and clinical practice has significantly impacted the management of cancer. Progressing from the detection of gene alterations and data analysis to actionability, targeted and whole-genome NGS has resulted in a better understanding of cancer genetics and its potential impact on patients' outcomes. In alignment with the broader landscape of cancer research and discoveries, colorectal cancer (CRC) has benefited from this breakthrough. Some treatments rely on various recent findings based on NGS-enhanced discoveries. However, cost, technical considerations, clinical validation, and other important issues limit its application in low-middle income countries. In this chapter, we discuss the challenges facing these advanced and tremendously improved technologies before their employment in routine laboratory practice.

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**Keywords**

Colorectal cancer · Genetics · Next-generation sequencing · Cost-effectiveness

## 7.1 Introduction

The arrival of NGS and a wide battery of genomic assays for screening, prognosis, and therapy resistance prediction have radically changed our perspective in personalized oncology (Nagarajan et al. 2017; Karlovich and Williams 2019; Morash et al. 2018). To better manage treatment decisions, several panels for genomic testing are used in clinical practice. This contribution has considerably improved clinical outcomes in several cancers, including pan-RAS wild-type colorectal cancer (CRC) (Vasconcellos et al. 2018). In fact, the percentage of patients who have benefited from genomic-driven cancer care in the USA has risen from 0.70% in 2006 to 4.90% in 2018, emphasizing a notable growth of targeted therapies approved by the FDA (Marquart et al. 2018). This growth is mainly attributed to considerable large-scale sequencing efforts that have been made to satisfy the missing links between genomic alterations and cancer treatment. The NGS cost is attractive when compared to traditional Sanger sequencing, which is still broadly used as the gold standard for detecting and validating genetic variants associated with various cancers (Mantere et al. 2019). NGS techniques have gained more attention, as they provide a deeper understanding of cancer genetics, rapid turnaround time and clinical reporting, as well as improved accuracy parameters (Sikkema-Raddatz et al. 2013), providing an affordable cost of $1000 for the whole-genome sequencing process (Hayden 2014; Nimwegen et al. 2016). Unfortunately, these advances in sequencing may present some barriers regarding cost-effectiveness, technical and regulatory barriers, and ethical considerations. In fact, besides an undeniable benefit to research into the understanding of cancer biology, clinical reports of genetic alterations will expose clinicians and then patients to diverse issues. These are related to a lack of knowledge on the role of these alterations on prognosis and treatment decisions. In low-middle income countries, at reduced economical levels, these limits should be considered before implementing NSG on a large scale.

## 7.2 Cost-Effectiveness of Next-Generation Sequencing in Colorectal Cancer

The cost-effect evaluation of NGS panels is based routinely on available data from clinical trials and merged financial data using a Markov model analysis—a powerful mathematical approach for modeling medical decisions and health economics (reviewed elsewhere: Komorowski and Raffa 2016) (Goldstein et al. 2015). Economic and clinical data are commonly presented in relation to the impact on life years gained or lost and then adjusted for the disease-related disability or quality of

life, producing cost estimates per quality-adjusted life years (QALYs) or disability-adjusted life year (DALY) (Komorowski and Raffa 2016; Tan et al. 2018). To assess the economic significance of genetic testing in CRC treatment, Behl et al. analyzed the cost-effectiveness of anti-EGFR treatment by comparing four strategies. These included "best supportive care," "anti-EGFR therapy without genetic screening," "screening for *KRAS* mutations only," and "screening for *KRAS* and *BRAF* mutations" (Behl et al. 2012). *KRAS* and *BRAF* testing was found to improve the OS by 0.0034 years at a cost of $22,033 with an incremental cost-effectiveness ratio of approximately $650,000 per additional year of life (Behl et al. 2012). However, in high-income countries, this increase is much greater than the accepted threshold for the cost-effectiveness ratio of $100,000/quality adjusted life year. The addition of *KRAS* and *BRAF* testing was found to be cost saving ($7500 and $1023 per patient, respectively) in this setting (Behl et al. 2012). This is explained by the fact that the indiscriminate treatment of all metastatic CRC patients would result in inappropriate anti-EGFR therapy for a large part of the population, with related investments in ineffective and potentially harmful targeted therapies (Behl et al. 2012). Gallego et al. performed a cost-effectiveness study of NGS panels used for CRC and polyposis syndrome diagnosis. This included Lynch syndrome and other high penetrant genes where patients were referred for medical genetics counseling (Gallego et al. 2015). Evaluation using this NGS panel was found to provide significant cost-effective clinical benefits (Gallego et al. 2015). In fact, an average increase of 0.151 year of life, 0.128 QALYs, and $4650 per patient was observed, and therefore, a differential cost-effectiveness ratio of $36,500 per QALY was achieved, compared to the standard of care (Gallego et al. 2015). Moreover, adding low penetrant CRC genes to this panel led to an incremental cost-effectiveness ratio of $77,300 per QALY (Gallego et al. 2015). In a German study, a single whole-genome sequencing analysis was found to exceed the promising "US$1000 per genome" threshold by more than a factor of 3.8 (Plöthner et al. 2017). The cost of NGS using Illumina HiSeq 2500 was estimated at 3858.06 € compared to Illumina HiSeq Xten which was less expensive (1411.20 €) (Plöthner et al. 2017). Similarly, a Dutch study assessed the cost of Illumina NextSeq 500-, HiSeq 4000-, and HiSeq X5-based sequencing in clinical practice. They found that per sample, whole-genome sequencing costs 1669 € and whole-exome sequencing and targeted panels were considerably lower (792 € and 333 €, respectively) (Nimwegen et al. 2016). Recently, an encouraging real-world study was carried out by a multicentric French group. They investigated the total cost of NGS in a diagnostic setting. This estimate would cover costs for pre-analytical steps, reporting of results to clinicians, and post-sequencing procedures (Marino et al. 2018). Contrary to expectations, detection of somatic mutations using targeted NGS panels costs 607 € (±207) and 555 € (±140) per patient for germline genetics (Marino et al. 2018). As expected, the enrichment phase where DNA libraries are generated was found to be the most cost-consuming (somatic genetics: 29% and germline genetics: 34% of the cost), while the sequencing phase costs only 20% and 9% of the total cost for somatic and germline analysis, respectively (Marino et al. 2018) (see Fig. 7.1). Therefore, the cost of NGS per

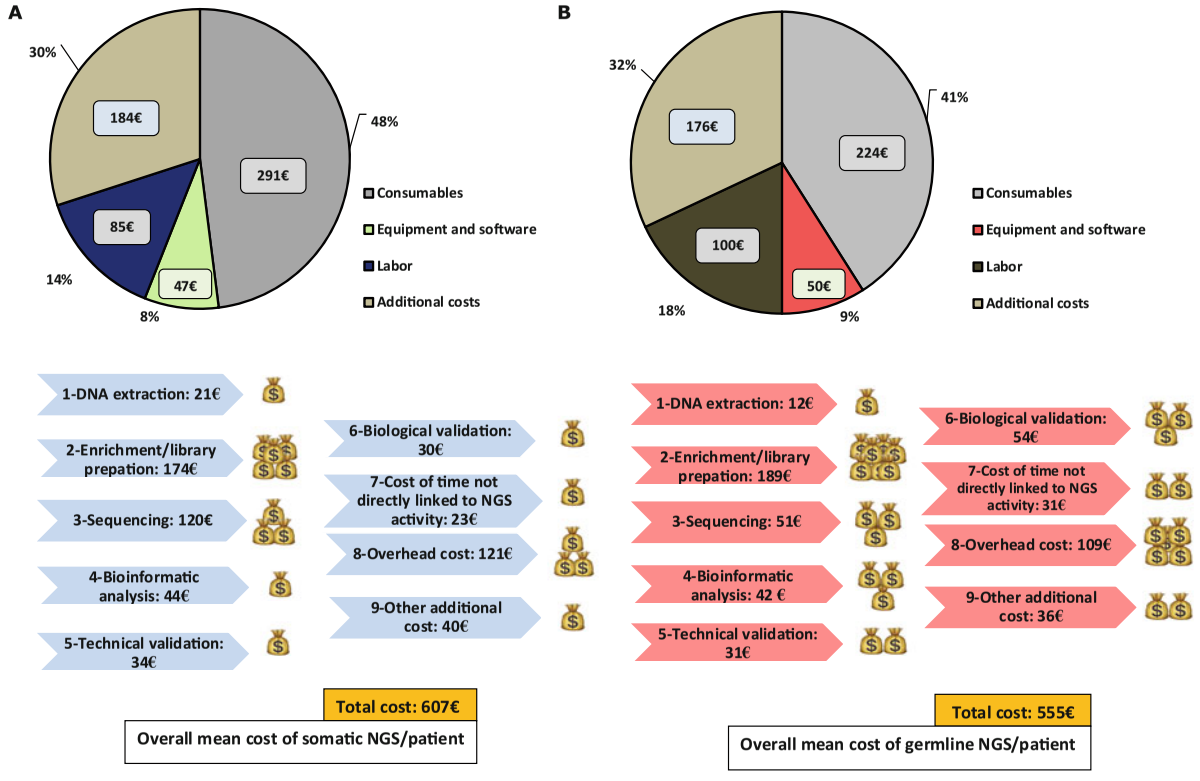


**Fig. 7.1** Overall mean cost per patient (in euro) of the NGS applications for targeted genes panel in (**a**) somatic and (**b**) germline sequencing analysis (data from: Marino et al. 2018)

patient is expected to decrease more in the near future with the advent of competitive companies and sequencing technologies.

## 7.3 Technical Considerations

### 7.3.1 Limitations of Next-Generation Sequencing

More recently, despite the considerable advantages of NGS, a number of limitations exist in relation to the technologies and/or their uses in clinical practice. Some limitations are inherent to the technologies themselves, and others are due to disease features and the analysis workflow. Detection of variants in tumoral tissues is challenging and depends on multiple parameters such as specimen type, liquid or solid tumor biopsies, heterogeneity and cellularity of tumors, storage, normal reference specimens, as well as downstream bioinformatic analysis (Jennings et al. 2017).

#### 7.3.1.1 Sampling and Library Preparation Bias

Sample acquisition in cancer could represent the first bias concerning NGS profiling of tumors. This bias is due to tumor heterogeneity, contamination with normal cells, low tumor cellularity, as well as the spatial and temporal tumorigenic process (Yap et al. 2012; LeBlanc and Marra 2015; Zheng et al. 2016). Multiple clones and sub-clones with different mutations and chromosomal abnormalities are found within the same tumoral specimen, leading to complex genomic profiling (Yap et al. 2012). Sample conservation is another parameter that affects the quantity and quality of DNA used in the NGS analysis (Zheng et al. 2016). Fixation and embedding of DNA for histopathological analysis alter DNA. When possible, extracting a sufficient quantity of DNA before tumor processing is the better alternative for genetic profiling by NGS (Zheng et al. 2016; Sone et al. 2019). In clinical practice, nucleic acids are more commonly extracted from formalin-fixed, paraffin-embedded tissue (FFPE) samples used for histopathological analysis. These nucleic acids create other biases to PCR duplicates, therefore increasing false-positive and false-negative mutation calls (Do and Dobrovic 2015; Gray et al. 2015). Low amounts and quality of DNA are both important factors that increase PCR duplicates and consequently error rates, especially false-positive errors (Gray et al. 2015). Fresh or fresh-frozen tumors remain the best solution for obtaining high-quality DNA or RNA for whole-exome sequencing (WES), whole-genome sequencing (WGS), transcriptomics, and optimal variant detection in tumoral tissues (Jennings et al. 2017; Müllauer 2017). Before library preparation, multiple steps of quality control are necessary. At this point, other biases can be detected because of low input DNA. In addition, GC-rich, AT-rich sequences, repeat regions, and regions with high homology present important issues during library preparation steps. Amplification of these genomic regions is still problematic and being improved in NGS platforms (Oyola et al. 2012; Chen et al. 2013). These complex genomic regions generate other issues during bioinformatic analysis steps (genome assembly and variant calling errors) (Oyola et al. 2012; Chen et al. 2013; Jennings

et al. 2017). Limitations raised by the amplification of these regions could be partially resolved using WGS—PCR-free amplification—or similar PCR-free protocols. However, a large amount of DNA/RNA is needed for this process, which is not suitable for tumor profiling using biopsy samples (Oyola et al. 2012). Moreover, the cost of WGS, important computer resources, and the complex pipelines for bioinformatic analysis, as well as storage issues required for WGS data analysis, limit its wide use for clinical diagnosis (Dove et al. 2015; Gray et al. 2015; Kamps et al. 2017).

#### 7.3.1.2 Sequencing, Bioinformatic Analysis, and Data Storage

In massively parallel sequencing technology platforms, errors in sequencing are ten times higher compared to Sanger sequencing (Gullapalli et al. 2012). Average error rates are estimated to be between 0.1% and less than 1% for sequencing by synthesis and are observed in single-nucleotide substitutions and indels (Pfeiffer et al. 2018; Morganti et al. 2019). These rates are higher for single-molecule real time (SMTR) (5–15%) and are not used yet for clinical diagnosis (Morganti et al. 2019). Polymerase, laser, CG and AT content, as well as sequencing technologies used in NGS machines contribute substantially to these sequencing errors (Oyola et al. 2012; Pfeiffer et al. 2018). In short read NGS technologies, these errors include nucleotide substitutions and indels which are seen at the end of reads (Ulahannan et al. 2013). Duplicate PCRs are also common in NGS due to library construction and are secondary to errors in sequencing, which inflate the average coverage (Gray et al. 2015; Zhang et al. 2019). In bioinformatic analysis, these errors generated by sequencing are taken into consideration. This is achieved by removing duplicate PCRs and sequences with high allele mutation frequency to obtain the real average coverage and reduce false-positive rates (Gray et al. 2015). However, removing these errors may lead to omitting somatic variants in cancer samples with low allele frequency (5%) (false negatives) (Gray et al. 2015; Ebbert et al. 2016).

Detecting variants is the critical step of NGS tumor profiling. Multiple factors can impact variant detection, and these include the use of fresh or fixed specimen samples, heterogeneity of the tumors, and the coverage and sensitivity of the bioinformatic tools used (Jennings et al. 2017; Alekseyev et al. 2018). Thereby, analyzing pairs (blood/tumor) or other normal tissues is recommended, because the use of tumors only can lead to missing actionable germline mutations classified as somatic (Gray et al. 2015). At the bioinformatic analysis step, lowering the limit of sensitivity in the genomic regions with these specific variants could help in detecting clinically significant variants with low variant allele frequencies (Jennings et al. 2017). Even if NGS technologies are the best approach to explore the heterogeneity of tumoral tissues, a deep average coverage (100×–1000×) is necessary to decipher this heterogeneity (Gullapalli et al. 2012; Xuan et al. 2013; Kamps et al. 2017; Alekseyev et al. 2018). The aim of using high coverage is to confidently detect a minor allele frequency of 5%, and an average coverage of 500×–1000× is recommended (Gray et al. 2015; Alekseyev et al. 2018). For the detection of germline mutations, coverage of around 30× is sufficient for variant detection (Jennings et al. 2017; Alekseyev et al. 2018). Assessment and interpretation of

detected variants and their classification should be performed according to the American College of Medical Genetics and Genomics (ACMG) standards and guidelines (Bahcall 2015; Richards et al. 2015). Classification of variants is considered as an important support tool for "medical decision-making" or "individualized treatment decisions" when variants and/or genes are actionable (Richards et al. 2015). However, variants with unknown significance (VUS) in genes known as pathogenic or in genes with unknown significance (GUS) remain a central issue in clinical applications of NGS and should not be considered in the decisions of health providers (Richards et al. 2015). In addition, to reduce turnaround time and costs, the use of targeted gene panels is recommended in cancer and allows for the detection of a wide number of actionable variants with high confidence for somatic mutations and reduced VUS and GUS (Richards et al. 2015; Paolillo et al. 2016). However, the use of gene panels and exome sequencing does not allow for structural variations (SVs) and/or copy-number alteration (CNAs) identification. These genomic variants are detected only by WGS, comparative genomic hybridization (CGH) array, or high-density single-nucleotide polymorphism (SNP) arrays which have important challenges due to the high heterogeneity and instability of tumoral tissues (Jennings et al. 2017; Nakagawa and Fujita 2018). WGS gives a broad view of genomic and genetic variations, but is limited by the important computational resources required for the analysis and interpretation of identified variants, SVs and CNAs with unknown significance (Berger and Mardis 2018; Nakagawa and Fujita 2018). The need for adequate storage facilities, time, and resources for analysis are more crucial and complex (Horak et al. 2016; Yin et al. 2017). NGS technologies generate billions of reads, representing a challenge for the transfer and storage of data (Metzker 2010; Reuter et al. 2015). Emerging commercial platforms provide multiple services for sequencing and data management which raises concern for the ethical aspects and privacy of human genomic data (Koboldt et al. 2013). Notably, targeted sequencing workflow seems to reduce both the cost and complexity of analysis as compared to WGS which until now has been recommended for clinical practice (Horak et al. 2016; Berger and Mardis 2018).

### 7.3.2 Next-Generation Sequencing in Low-Middle Income Countries

In developing countries, a number of parameters limit the implementation of NGS facilities for research and clinical testing (Helmy et al. 2016). The major limit of establishing such facilities is the cost of the equipment and need for a highly specialized workforce. Costs are often higher in low-middle income countries because of the involvement of several intermediate brokers, a common issue of the healthcare market (Helmy et al. 2016). The lack of trained molecular and clinical geneticists and bioinformaticians in NGS is challenging in Africa. It constitutes one of the key problems facing its rapid implementation (Mlotshwa et al. 2017). Consequently, minimal representation of developing countries and especially Africa was seen in genomic research (Mulder et al. 2016; Mlotshwa et al. 2017). For instance,

the H3Africa Initiative (https://h3africa.org/) founded by National Institutes of Health (NIH) and the Wellcome Trust foundation support the development of genomic research and implementation of facilities for research in genomic medicine as well as genomic analysis training for African scientists. This initiative also includes a bioinformatics network (H3ABioNet, https://www.h3abionet.org/) which was specifically built to enable sustainable genomic data analysis for African researchers across the continent (Mulder et al. 2016). Genetic studies in populations and cancer patients in Africa can improve our understanding of susceptibility to risk factors, patient prognosis, and benefits from treatment. Therefore, we may then be able to reduce our doubts relating to blind acceptance of evidence based on non-African populations. Other initiatives such as the Collaborative African Genomics Network (CAf-GEN) have recently emerged to train African scientists for genomic research and clinical testing and to build local research and clinical genomic centers (Mlotshwa et al. 2017). Regardless of the lack of potential initiatives, often at the pilot phase for feasibility assessment, this is an ideal opportunity to build appropriate resources for African medical geneticists and bioinformaticians. The goal is to train a research-oriented workforce and have key human resources, who will then analyze clinical genetic data which will in turn provide genetic services for low-middle income countries. However, these programs are not yet satisfactory considering the important advances in personalized oncology. Concentrated effort is needed in order to develop the field of medical genetics within the context of healthcare providers in developing countries.

## 7.4 Conclusions and Perspectives

NGS is revolutionizing medical practice as personalized medicine becomes more and more relevant. Advances in NGS have enabled a better understanding of disease mechanisms, cancer diagnosis, prognostic stratification, and personalized treatments (Hux et al. 2019). Decreased costs of these novel technologies have contributed significantly to progress in this field. However, only modest development in low-middle income countries is evident at this time (Helmy et al. 2016). Targeted sequencing would be the best cost-effective option for clinical practice; it could be suitable for developing countries having limited resources. Globally, NGS has raised several issues concerning handling human genomes. This includes technical limits, storage, transfer, analysis, and patient data privacy (Reuter et al. 2015). In addition, incidental findings remain an important limit in clinical testing using NGS (Hux et al. 2019). Reporting these findings is continually debated, especially in the case of variants or genes with unknown significance (Richards et al. 2015; Hux et al. 2019). Clinicians should be informed as to how to properly report these variants to patients in order to accurately improve outcomes.

In summary, the rapid progression of NGS technology has illuminated the actionable genetic alterations of CRC and provided a deeper understanding of the genetic and epigenetic hallmarks of the disease. NGS technology has (1) powered our comprehension of sporadic and hereditary CRC genetics and its significant

application as a potential diagnostic, prognostic, and predictive biomarker, (2) appropriately facilitated the development and application of a liquid biopsy in CRC management, (3) unraveled the possible causative link between the gut microbiome and CRC, and (4) propelled single-cell genomics (fourth-generation sequencing) which is revolutionizing the field of CRC genetics (Ke et al. 2016; Baslan and Hicks 2017). In conclusion, implementing NGS in clinical patients' care should be goal oriented. Such technologies require additional assessments regarding standardization, big data interpretation, and privacy. Clinicians and geneticists should be trained to manage whole NGS workflow to improve patients' outcomes. We believe this book chapter (and other information in this book) addresses many of the challenges and new approaches regarding the management of CRC. Our future expectations include a decrease in the costs for NGS, anticipating the advent of competitive technologies to shape the market and reduce monopolies. A better shift in the advances of NGS, especially in building companion diagnostics based on targeted sequencing panels, seems to be the best opportunity for molecular testing (supportive data can be found in Box 7.1). Unresolved issues regarding high-throughput NGS applications in clinical practice and research are on the rise. These include problems relative to data storage and bioinformatic pipelines, the lack of a highly skilled and trained staff, and high cost of consumables. Despite all of these efforts, NGS technologies are so far affordable, especially in the context of developing countries that have limited resources. Their role in CRC is still evolving and merits further development. Globally, improvement in cancer care is still complex and remains a major challenge of modern oncology.

**Box 7.1**

| Recommended reading | DOI |
|---|---|
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(continued)

**Box 7.1** (continued)

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|---|---|
| Sabatini LM, et al. *Genomic Sequencing Procedure Microcosting Analysis and Health Economic Cost-Impact Analysis: A Report of the Association for Molecular Pathology*. J Mol Diagn. 2016;18(3):319–328. | https://doi.org/10.1016/j.jmoldx.2015.11.010 |
| van Nimwegen KJ, et al. *Is the $1000 Genome as Near as We Think? A Cost Analysis of Next-Generation Sequencing*. Clin Chem. 2016;62(11):1458–1464. | https://doi.org/10.1373/clinchem.2016.258632 |
| Plöthner M, et al. *Cost-Effectiveness of Pharmacogenomic and Pharmacogenetic Test-Guided Personalized Therapies: A Systematic Review of the Approved Active Substances for Personalized Medicine in Germany*. Adv Ther. 2016;33(9):1461–1480. | https://doi.org/10.1007/s12325-016-0376-8 |
| Jain S, Shankaran V. *The economics of personalized therapy in metastatic colorectal cancer*. Curr Colorectal Cancer Rep. 2016;12:123–9. | https://doi.org/10.1007/s40273-018-0619-4 |

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