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REVIEW

Somatic pharmacogenomics in the treatment prognosis of locally advanced rectal cancer patients: a narrative review of the literature

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ABSTRACT

Introduction: Standard treatment for patients with locally advanced rectal cancer (LARC) includes neoadjuvant chemoradiotherapy (nCRT) with fluoropyrimidines, followed by surgical excision. The newly introduced therapeutic strategies propose intensified regimens or more conservative approaches based on risk stratification algorithms that currently include clinicoradiological criteria but not molecular variables. How to better stratify patients is a burning clinical question, and pharmacogenomics may prove useful in identifying new genetic markers that could be incorporated into clinical algorithms to personalize nCRT. An emerging area could be the evaluation of somatic mutations as potential genetic markers that correlate with patient prognosis. Tumor mutations in the *RAS/BRAF* genes, as well as microsatellite instability (MSI) status, are currently used in treatment selection for colorectal cancer (CRC); however, their clinical value in LARC is still unclear.

Area covered: This literature review discusses the relevant findings on the prognostic role of mutations in the key oncogenes *RAS, KRAS, BRAF, PIK3CA, SMAD4* and *TP53*, including MSI status in LARC patients treated with nCRT.

Expert opinion: *KRAS* proved to be the most promising marker, consistently associated with poorer disease-free survival and overall survival. Therefore, *KRAS* could be a good candidate for integration into the risk stratification algorithm to develop a personalized treatment.

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KEYWORDS
KRAS; locally advanced rectal cancer; MSI; neoadjuvant chemoradiotherapy; personalized treatment

1. Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second leading cause of cancer death worldwide [1]. Rectal cancers account for about half of CRCs, and their incidence is increasing exponentially, especially in individuals under 50 years of age [2,3]. A significant proportion of patients with rectal cancer present with locally advanced rectal cancer (LARC), which is defined by the presence of T3/T4 carcinoma with or without lymph node infiltration (N0/N+) and without metastatic spread [4]. For a long time, the mainstay treatment for LARC patients was a multimodal approach involving the use of fluoropyrimidine-based neoadjuvant chemoradiotherapy (nCRT) followed by total mesorectal surgical excision [5,6]. Postoperative adjuvant chemotherapeutic treatment could also be considered after radical surgery to reduce the risk of disease recurrence depending on clinical judgment [5,6]. Despite the improvement in clinical outcomes with conventional therapy, the high rate of distant metastases (30–35%), the low pathological complete response (pCR) (10–25%) and the very inconsistent adherence to adjuvant chemotherapy (25–75%) are still open questions [7]. Moreover, the majority of patients develop severe symptoms and morbidities associated with surgical resection [7]. To overcome these limitations and improve prognostic outcomes, new treatment modalities for LARC have been established in recent years. In particular, for patients at high risk of recurrence, intensified preoperative therapies such as the total neoadjuvant treatment (TNT) are proving to be an ideal strategy with the aim of improving pCR rates, increasing treatment adherence, treating micrometastasis earlier and reducing distant recurrence [8,9]. On the other hand, in patients at low and intermediate risk of recurrence who achieve a complete or near-complete clinical response, organ-preserving approaches such as local excision (LE) or the watch-and-wait strategy could be considered, aiming to avoid surgery and long-term postoperative morbidities while maintaining adequate disease control [9,10]. The ability to stratify LARC patients based on risk of local recurrence and long-term outcomes may therefore be an important guide for clinical decision-making and personalization of therapeutic strategies. Currently, risk stratification of patients is mainly based on clinicopathological criteria (i.e. clinical T and N stages, distance of the tumor from the anal verge, involvement of the mesorectal fascia, extramural vascular invasion) [10–13] and more recently also on radiomic features [14], although no molecular markers are taken into account. Considering the still high recurrence rate an improvement of the risk stratification algorithm is certainly necessary to allow a better selection of the most appropriate therapeutic

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Article highlights

- In recent years, choosing the most appropriate therapeutic approach based on patient risk stratification has become a crucial step in the treatment of LARC patients.
- Several pharmacogenomic studies have investigated the role of somatic mutations in key oncogenes in predicting the risk of local recurrence/distant metastasis and survival probability in LARC patients.
- *KRAS* mutations represent a promising biomarker that, once validated by future prospective clinical trials, could be included in the decision algorithm to select the most appropriate treatment strategy for LARC patients (e.g intensified therapy or organ-preserving approaches).
- Due to their heterogeneity, only a trend for better DFS in patients with dMMR tumors could be derived from the articles examined.
- For the other genes investigated (i.e. *TP53, BRAF, PIK3CA* and *SMAD4*), the small number of published studies together with the heterogeneity of the data obtained does not allow a definitive conclusion on a possible role of mutations in these genes.

strategy, with a positive impact on patients' prognosis [15–19]. Therefore, the discovery of additional novel molecular predictors that can be integrated into clinical practice could be useful to improve the management of LARC patients. In this context, pharmacogenomics could be a useful strategy to personalize and optimize nCRT in LARC [20].

Rapid advances in genetic and molecular biology have led to the realization that somatic mutations play an essential role in the development and prognosis of rectal cancer and in influencing the modulation of therapy outcome [20–22]. In CRC testing for tumor mutations in key oncogenes (i.e. *RAS* and *BRAF*) and microsatellite instability (MSI) status is required for the selection of the most appropriate treatment [23,24]. However, with the exception of MSI characterization, which has recently been introduced with the advent of immunotherapy, molecular markers in LARC are still not included in the risk algorithms used in clinics. The spectrum of molecular tumor alterations and the mechanism of oncogenesis have been reported to be remarkably different between colon and rectal tumors [25–27]. Even though 82% of non-metastatic rectal cancers display mutations in cancer driver genes belonging to the PI3K and MAPK signaling pathways (e.g. *KRAS, PIK3CA, TP53*) [28] similar to colon cancer, the distribution of these alterations was significantly different between rectal and colon tumors [25,27]. For example, mutations of the RAS and PI3K pathways were more frequent in colon carcinomas, whereas alterations of the TP53 pathway were more frequent in rectal carcinomas [25,27]. Similarly, SMAD4 mutations were significantly more frequent in rectal cancer compared to distal colon cancer [29]. Therefore, pharmacogenomic data obtained in CRC cannot be automatically transferred to the treatment and management of non-metastatic rectal cancer. The predictive and prognostic role of *KRAS* and *TP53* mutations has been extensively investigated in LARC patients treated with nCRT [20], but their clinical value in this setting remains uncertain due to the large heterogeneity of published data. Other genes investigated in rectal cancer with contrasting results include *BRAF, PIK3CA, SMAD4* and tumor MSI [20,30].

In the present review, we focus on MSI status and somatic alterations in genes (i.e. *RAS*, *TP53*, *BRAF*, *PIK3CA* and *SMAD4*) that are frequently mutated in rectal cancer and that have been investigated for their potential role in influencing the outcome of neoadjuvant radiation-based therapy in patients with LARC. We have previously analyzed the role of these molecular markers in predicting the risk of non-response to pre-operative treatment in LARC patients (no pCR) [31]. In the present study, we conducted a revision of literature to summarize the published data on the prognostic value of the same molecular markers and their impact on the disease-free survival (DFS) and overall survival (OS) (Figure 1). Given the multiple therapeutic options available today for the treatment of LARC patients, the identification of new prognostic markers could help clinicians to select the most appropriate therapeutic strategy and thus improve the oncologic outcome and quality of life of patients by avoiding under- or over-treatment.

2. Materials and methods

A literature search was performed for all published studies addressing the impact of mutations in candidate genes (i.e. *RAS, TP53, BRAF, PIK3CA* and *SMAD4*) or MSI status on prognosis (i.e. local/distant recurrence risk and survival) after nCRT or radiotherapy (RT) in LARC patients. The PubMed Database was searched for relevant articles published in English in a peer-reviewed journal from inception through 1 December 2023. The search algorithms included the keywords 'rectal cancer,' 'chemoradiotherapy' or 'radiotherapy,' and 'candidate gene' or 'MSI,' combined with Boolean operators (OR/AND). Additional studies were identified by hand-searching the references of relevant articles. A total of 290 studies published between December 1997 and September 2023 were retrieved. Inclusion criteria were that the studies were published in English in a peer-reviewed journal and contained data on the topic of the present review. The molecular data should be obtained from tumor tissue samples. Reviews and case reports were excluded from the eligible articles. The flowchart of the literature search is shown in Figure 2 Finally, 43 studies [28,32–73], published between December 2000 and September 2023, were selected and discussed in the present review that addressed the ability of somatic mutations in the *RAS, TP53, BRAF, PIK3CA*, and *SMAD4* genes and MSI status to predict prognostic outcomes in LARC patients who received RT-based preoperative treatment. For each eligible study, the following items were recorded: the mutations or markers tested, the method of molecular analysis, the tumor tissue source in which the molecular data were obtained, the characteristics of the study population (number of patients and country of origin), the patient enrollment interval, the tumor stage, the therapeutic strategy including possible adjuvant treatment and the chemotherapeutic agent used, the clinical endpoint investigated, and the main outcomes (Tables 1 and 2).

3. Results and discussion

3.1. All RAS

RAS proteins are ubiquitously expressed in all mammalian cells and there are three isoforms: *H-Ras*, *K-Ras* and *N-Ras*, which are characterized by a high degree of similarity. They are all involved in the regulation of relevant cellular pathways such as proliferation and survival, so that single mutations in

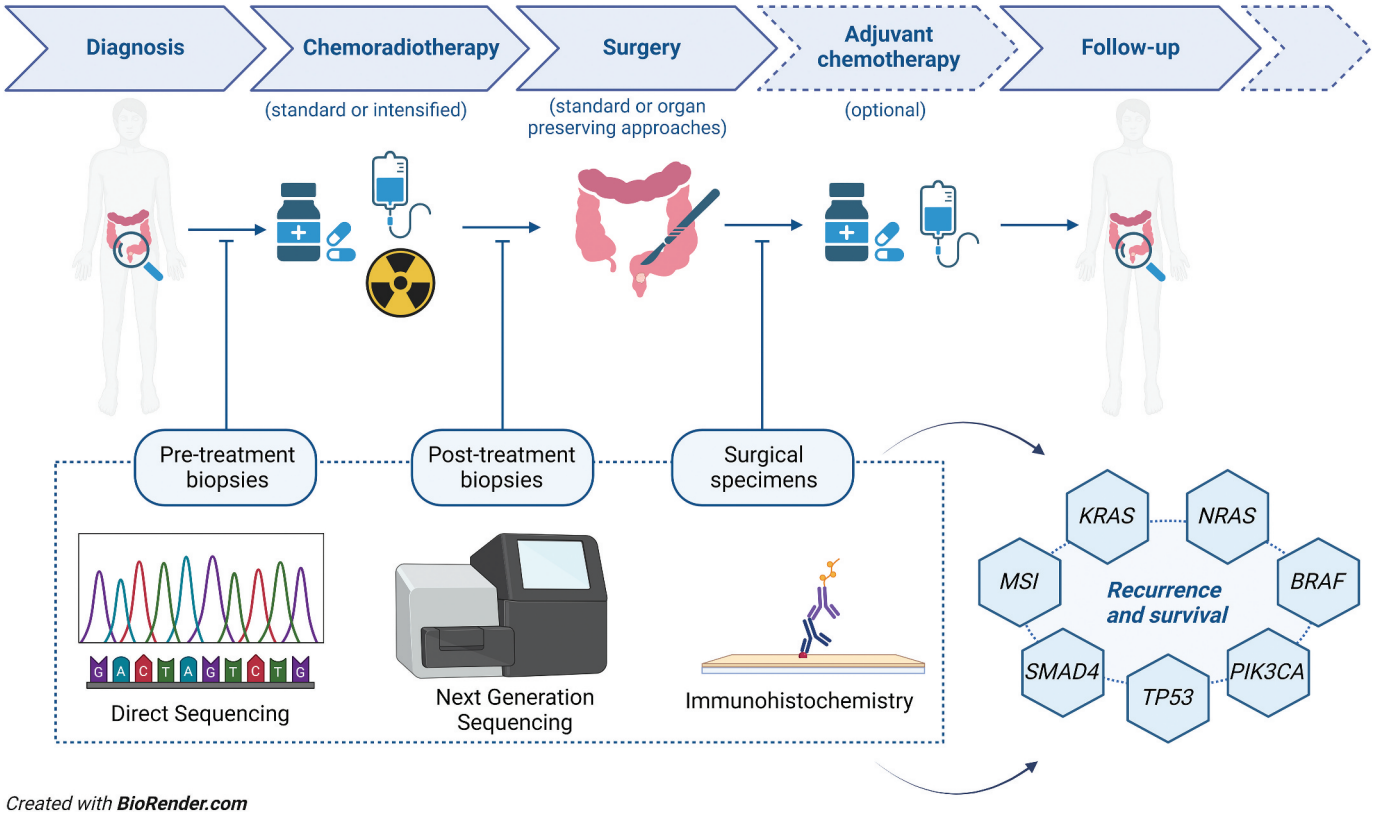

Figure 1. Representative illustration of the aim of this literature review which summarizes the published data on the prognostic role of mutations in the major oncogenes RAS, BRAF, PIK3CA, SMAD4 and TP53, as well as MSI status and evaluates their impact on disease-free survival (DFS) and overall survival (OS) in LARC patients treated with nCRT. Figure created with BioRender.com.

these genes, which typically occur at codons 12, 13 or 61, are responsible of oncogenesis promotion. The impact of *RAS* mutations in colon cancer is clear, but the contribution of these same mutations in rectal cancer needs to be better elucidated [74,75].

Four eligible articles [32–35] were found that focused on the entire RAS signaling pathway and examined the prognostic value of mutations on the *KRAS* and *NRAS* genes, while no article included mutations on the *HRAS* gene in the analysis. This could be due to the fact that *KRAS* is the most frequently mutated isoform in almost 40% of all CRCs, followed by *NRAS* (4%), while mutations on the *HRAS* gene are very rare, accounting for less than 2% of cases [76]. Specifically, within the RAS family, *KRAS* mutations represents approximately 85% of all *RAS* mutations in human tumors, while *NRAS* mutations account for about 15% and *HRAS* mutations for less than 1% [77]; this is the reason why the results of the studies analyzing the mutational status of *RAS* genes are mainly driven by the contribution of *KRAS* variations.

3.1.1. Recurrence risk

Four studies investigated the impact of *RAS* mutations on recurrence rates by assessing different endpoints (i.e. DFS; progression-free survival, PFS; and recurrence-free survival, RFS) [32–35] (Table 1(a)). These works, which had a small sample size overall (i.e. less than one hundred patients), were characterized by homogeneity in terms of the clinical stage of the patients at diagnosis (II or III), with the exception of Peng et al. [33] where this information was not available, but by quite a bit of heterogeneity in the other clinical characteristics, especially in terms of the preoperative treatment received by the patients. In the analysis by Gollins et al. [32], there was no difference in PFS depending on *RAS* mutational status ($p = 0.079$). It should be noted that the neoadjuvant treatment adopted in this study included cetuximab (CTX), irinotecan (IRI) and capecitabine (CAPE), a regimen that is no longer used today. However, similar results were obtained in the study by Sendoya et al. [34] which failed to demonstrate a correlation between RFS and the presence of *RAS* variants in patients treated with CAPE followed by RT with or without an intensified treatment regimen (e.g. TNT), which is the currently used neoadjuvant treatment regimen. In contrast, two studies in which patients were treated with standard nCRT based on fluoropyrimidines with or without oxaliplatin provided positive results [33,35]. In particular, Peng et al. [33] found an association between the presence of mutations on *RAS* genes and poor 3-year DFS ($p = 0.004$). Consistent with this finding, the more recent work by Orlandi et al. [35] confirmed the negative impact of *RAS* status on 5-year PFS ($p = 0.00039$).

3.1.2. Survival analysis

Three of the four eligible papers investigated the association between RAS mutations and survival [32,33,35] (Table 2(a)). In particular, Gollins et al. [32] reported that the presence of RAS mutations was not predictive of poorer survival ($p = 0.079$). In contrast, the other two papers found lower overall survival

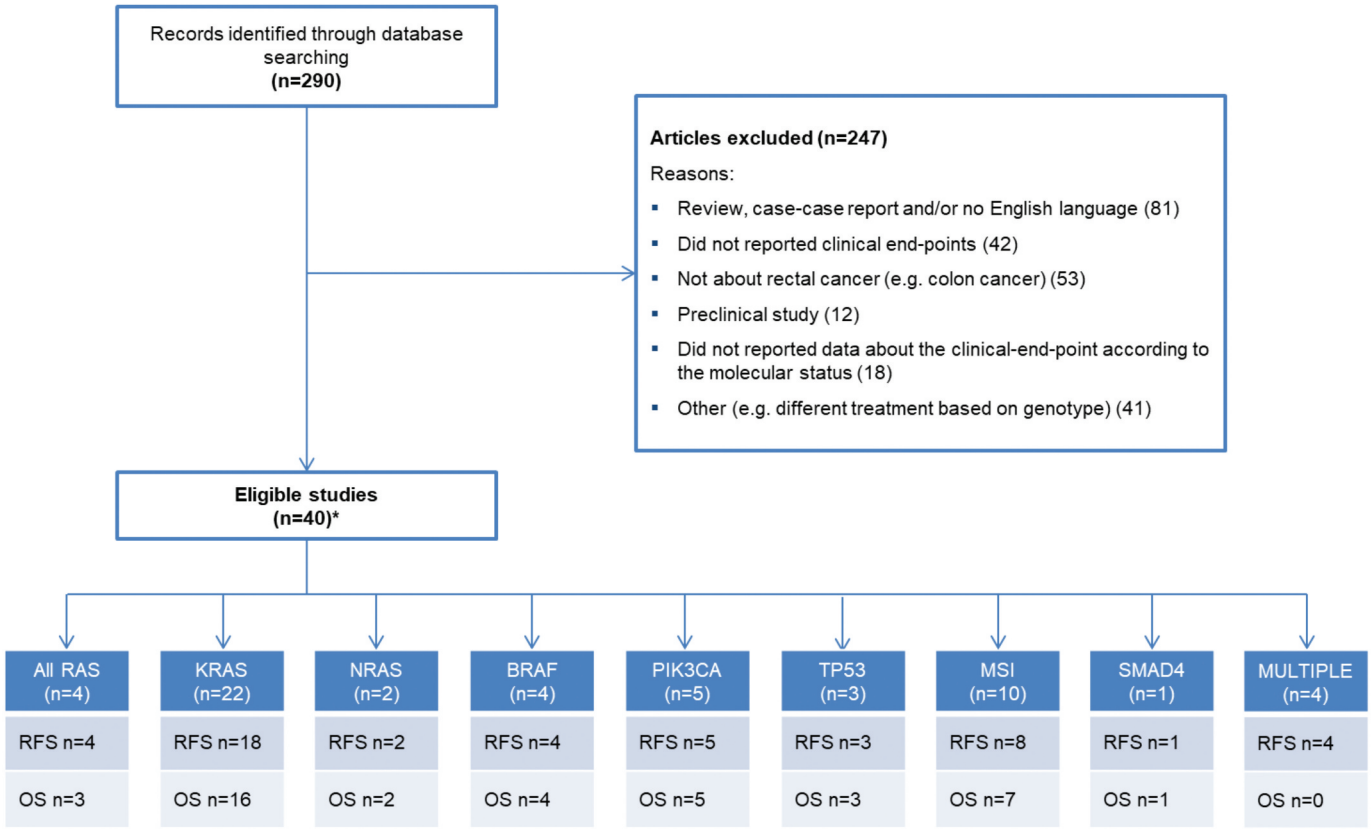

Figure 2. Flow chart that summarizes the results of the literature search.

*The number of eligible studies reported in the graph does not include the 3 meta-analyses. Some eligible studies investigate the role of more than one gene or more than one endpoint.

Abbreviations: RFS, recurrence-free survival; OS, overall survival.

(OS) in *RAS*-mutated patients than in *RAS* wild-type patients ($p = 0.036$ and $p = 0.0022$, respectively) [33,35].

3.2. KRAS

K-Ras mutations are among the most frequently observed and studied alterations in all types of cancer. This was also evident in our review, in which this gene was the most studied with 24 eligible articles (22 studies and 2 meta-analyses) addressing the prognostic impact of somatic *KRAS* alterations on treatment outcome (Tables 1(b) and 2(b)). As mentioned above, *KRAS* mutations were also responsible for the observed association between RAS status and recurrence risk or survival, as the mutation frequency of the *KRAS* gene is higher compared to the other *RAS* genes.

As shown in the literature, *KRAS* mutations are predominantly point substitutions in codon 12 (80.0%) and codon 13 (14.0%), while mutations in other codons (e.g. 61 and 146) are relatively rare [78]. This is also confirmed in our review, in which all included studies for this gene focused on the mutation hotspots G12 and G13 and in some cases extended the analysis to other additional hotspots (Tables 1(b) and 2(b)). Mutation analysis was mainly performed using sequencing-based methods (12/22 studies, 54.5%) (i.e. Sanger sequencing and pyrosequencing) and revealed a prevalence of *KRAS* mutations of 20% to 50%, confirming the frequency of about 40% reported for variations in this gene in colorectal cancer. The most common subtype is $KRAS^{G12D}$ (GGT > GAT) (30–60%), as reported in six studies included in our review [36,41,45,46,49,68].

3.2.1. Recurrence risk

Eighteen studies [28,33,36–50,52], most of which had a sample size of less than 100 patients (15/18, 83.3%), investigated the impact of *KRAS* mutations on recurrence risk, with a focus on the DFS endpoint (Table 1(b)). Overall, the articles were characterized by a high degree of heterogeneity in terms of study characteristics (e.g. stage at diagnosis, treatment regimens, biological matrix for molecular analysis) and results obtained. However, when the articles were clustered according to the publication date (before 2016 and after 2016), two groups of articles characterized by more homogeneous study characteristics and results can be identified. In particular, all studies published before 2016 (11/18, 61.1%) showed no association between *KRAS* status and recurrence rate, while studies published after 2016 (7/18, 38.9%) consistently showed a significant association between *KRAS* mutations and a higher risk of recurrence. These contrasting results could likely be due to the different characteristics of the studies conducted after or before 2016. The analysis performed before 2016 also included clinical stage IV (3/11, 27.3%), which could have a negative impact on patients' prognosis, affecting the possibility of finding a significant genotype-phenotype correlation. In contrast, the most recent studies (after 2016) limited the inclusion criteria to clinical stages I, II and III in order to

Table 1. Published works on the impact of somatic mutation in oncogenes and MSI status on recurrence risk in locally advanced rectal cancer (LARC) treated with preoperative radiation-based therapy.

Mutations or markers tested	Analytical method	Tumor Tissue Source	Study Population (n °/Country)	Enrollment Interval	Stage	Therapy strategy	CT agents*	Adjuvant Therapy*	Clinical End-point	Main findings	Ref
a) All RAS											
KRAS codons 12, 13, 61, 146; **NRAS** codons 12, 13, 61.	Pyrosequencing and NGS	FFPE pretreatment biopsies and surgical resection tumor tissues	n = 78, UK	April 2009–October 2011	II – III	CRT + surgery	CAPE, CTX and IRI	FL ($n = 23$) or FL +OXA ($n = 28$) and $n = 3$ unspecified regimen	PFS	Improvement in PFS for WT compared to RAS mutated cancers (HR 0.53, 95%CI: 0.23 to 1.22).	Gollins et al. [32]
KRAS (G12V, G12A, G12D, G12C, G12S, G12R, G12F, G13V, G13D, A59T, Q61E, Q61K, Q61L, Q61R, Q61P, Q61H, Q61H); **NRAS** (G12V, G12A, G12D, G12C, G12R, G12S, G13V, G13A, G13D, G13C, G13R, G13S, A18T, Q61L, Q61R, Q61P, Q61H, Q61E, Q61K)	Sequenom MassARRAY system – OncoCarta Array	FFPE pre-treatment biopsies	n = 70, China	July 2006–June 2012	II-III	CRT + surgery	XELOX	XELOX	3 yrs – DFS	RAS mutations were associated with lower DFS-3 yrs rate than RAS WT (65.5% vs 92.3%, $p = 0.004$).	Peng et al. [33]
All RAS (KRAS $n = 18$ G12S, G12A, G12V, G12D, G13D, T74P, A146T; NRAS $n = 3$)	NGS and Gene Read CRC Panel, 38 genes ($n = 42$)	Frozen and FFPE pretreatment specimens	n = 50, Argentina	November 2015–September 2018	na	nCRT + surgery ($n = 23$) or TNT + surgery ($n = 27$)	CAPE, for TNT CAPE and CAPOX	Yes, for some pts.	RFS	No significant association.	Sendoya et al. [34]
KRAS (codons 12, 13, 59, 61, 117 and 146). **NRAS** (codons 12, 13, 58, 59, 61, 117 and 146).	Pyrosequencing	Pre- or post-treatment specimens (formalin fixed and kerosene included)	n = 39, Europe (Italy)	May 2011–June 2017	II – III	nCRT + surgery	5-FU or CAPE	Yes, $n = 16$. XELOX or FOLFOX.	5 YRS – PFS	The 5-year PFS in the Kaplan – Meier curves was significantly different between RAS wild type and RAS mutated pts ($p = 0.00039$)	Orlandi et al. [35]
b) KRAS											
Exons 1–3, codons 12-13-61-146	Sanger Sequencing	FFPE pre-treatment biopsies	n = 94, Europe (Germany)	February 1995–February 2010	II-III	CRT + surgery	5-FU ($n = 57$) or 5-FU+OXA ($n = 37$)	na	DFS	No significant association.	Gaedcke et al. [36]
Codons 12–13	Sanger Sequencing	FFPE pre-treatment biopsies	n = 146, Europe (Italy)	May 1998–October 2005	II-III-IV	CRT + surgery	5-FU ($n = 98$), 5-FU+OXA ($n = 34$), CAPE ($n = 14$)	na	5 yrs – DFS	No significant association.	Bengala et al. [37]

(*Continued*)

Table 1. (Continued).

Mutations or markers tested	Analytical method	Tumor Tissue Source	Study Population (n °/Country)	Enrollment Interval	Stage	Therapy strategy	CT agents*	Adjuvant Therapy*	Clinical End-point	Main findings	Ref
Codons 12-13-61	Pyrosequencing	FFPE pre- or post-treatment biopsies	n = 67, USA	na	II-III-IV	CRT + surgery	5-FU	na	Local/distant recurrences	No significant association.	Davies et al. [38]
Codons 12-13-21 (Q21K) – 60 (Q60T)	Sanger Sequencing	FFPE pre-treatment biopsies	n = 57, Europe (Germany)	na	II-III	Intensified CRT + surgery	CTX + IRI + CAPE	na	3 yrs – DFS	No significant association.	Erben et al. [39]
Codons 12 -13-61 (G12D, G12V, G13D)	Sanger Sequencing	FFPE or fresh frozen pre-treatment biopsies	n = 38, Korea	May 2006–December 2006	II-III	CRT + surgery	CTX + IRI + CAPE	Yes, *n* = 37. 5-FU/LV or CAPE.	3 yrs – DFS	No significant association.	Kim et al. [40]
Codons 12–13	Pyrosequencing	FFPE pre-treatment biopsies	n = 63, China	September 2007–March 2008	II-III	CRT + surgery	CAPE + CTX	Yes, some pts. Unspecified regimen.	3 yrs – DFS	No significant association.	Sun et al. [41]
Codons 12–13	Pyrosequencing	FFPE pre-treatment biopsies	n = 63, China	September 2007–March 2008	II-III	CRT + surgery	CAPE + CTX	Yes, some pts. Unspecified regimen.	3 yrs – Local control	No significant association.	Sun et al. [41]
Codons 12–13	Procedure defined by Response Genetics Inc.	FFPE pre-treatment biopsies	n = 32, Europe (Slovenia)	February 2007–September 2008	II-III	Intensified CRT + surgery	**Pre RT**: CAPE followed by CTX. **CRT**: CAPE	CAPE	3 yrs – DFS	No significant association.	Velenik et al. [42]
Codons 12–13	Procedure defined by Response Genetics Inc.	FFPE pre-treatment biopsies	n = 32, Europe (Slovenia)	February 2007–September 2008	II-III	Intensified CRT + surgery	**Pre RT**: CAPE followed by CTX. **CRT**: CAPE	CAPE	3 yrs – RFS	No significant association.	Velenik et al. [42]
Exon 2 (e.g. codon 12: G12D, G12V, G12S, G12R, G12C; codon 13: G13D) and exon 3	Sanger Sequencing	FFPE pre-treatment biopsies	n = 98, Europe (France)	May 2006–September 2009	I-II-III	CRT + surgery	5-FU + LV or CAPE	na	Local/distant recurrence	No significant association.	Derbel et al. [43]
Exon 1 (codons 12–13) and exon 2	Sanger Sequencing	FFPE pre- or post-treatment biopsies	n = 48, Europe (Germany)	February 2005–March 2006	II-III-IV	CRT + surgery	CTX-CAPE-OXA	No (*n* = 17), 5-FU/LV or CAPE (*n* = 15), 5-FU/LV or CAPE+OXA (*n* = 20), RFA (*n* = 1).	DFS	No significant association.	Fokas et al. [44]
Codon 12 (G12A, G12C, G12D, G12S, and G12V) and codon 13 (G13D)	PCR assay	FFPE pre-treatment biopsies	n = 100, Korea	December 2008–September 2013	II-III	CRT + surgery	5-FU + LV	5-FU	3 yrs – RFS	No significant association.	Lee et al. [45]
Codons 12 (G12D, G12V) –13 (G13D) –59-61-117-146	Sanger Sequencing	FFPE pre- and post-treatment biopsies	n = 76, Europe (Italy)	2007–2012	II-III	CRT + surgery	5-FU	na	3 yrs – DFS	No significant association.	Martellucci et al. [46]

(*Continued*)

Table 1. (Continued).

Mutations or markers tested	Analytical method	Tumor Tissue Source	Study Population (n °/Country)	Enrollment Interval	Stage	Therapy strategy	CT agents*	Adjuvant Therapy*	Clinical End-point	Main findings	Ref
Codons 12 (G12D, G12V) –13 (G13D) –59-61-117-146	Sanger Sequencing	FFPE pre- and post-treatment biopsies	n = 76, Europe (Italy)	2007–2012	II-III	CRT + surgery	5-FU	na	Local recurrence	No significant association.	Martellucci et al. [46]
Codon 12 (G12D, G12V, G12A, G12C, G12S) and codon 13 (G13D)	Sanger Sequencing	FFPE pre-treatment biopsies	n = 63, Europe (Serbia)	2006–2010	II-III	CRT + surgery	5-FU + LV	na	Local/distant recurrence	Globally there is no significant association (p = 0.101). **Subgroup analysis: 1)** pts with tumor at 7 cm from the anal verge and KRAS mutations had more local and distant recurrences than those with WT KRAS (59% vs. 20%, p = 0.036); **2)** pts with KRASmut/highVEGF expression had more recurrence compared to the other (26% vs 75%, p = 0.003).	Krajnović et al. [47]
G12V, G12A, G12D, G12C, G12S, G12R, G12F, G13V, G13D, A59T, Q61E, Q61K, Q61L, Q61R, Q61P, Q61H, Q61H	Sequenom MassARRAY system – OncoCarta Array	FFPE pre-treatment biopsies	n = 70, China	July 2006–June 2012	II-III	CRT + surgery	XELOX	XELOX	3 yrs – DSF	KRAS mutations were associated with lower 3 yrs – DFS rate than KRAS WT (68% vs 88.3%, p = 0.016).	Peng et al. [33]
Codons 12–13	PCR assay	FFPE biopsies	n = 60, Egypt	March 2014–December 2015	I-II-III	na	na	na	5 yrs – DFS	Pts carrying KRAS mutation had a significantly reduced 5 yrs – DSF compared to WT pts (37.5% vs 80.6%, p = 0.004).	Bahnassy et al. [48]
Codons 12–13	PCR assay	FFPE biopsies	n = 60, Egypt	March 2014–December 2015	I-II-III	na	na	na	Distant recurrence	Pts with mutated KRAS developed more distant metastasis (14/24; 58.3%) compared to WT-KRAS pts (6/36; 16.7%) (p = 0.001).	Bahnassy et al. [48]

(*Continued*)

Table 1. (Continued).

Mutations or markers tested	Analytical method	Tumor Tissue Source	Study Population (n °/Country)	Enrollment Interval	Stage	Therapy strategy	CT agents*	Adjuvant Therapy*	Clinical End-point	Main findings	Ref
Exon 2: codon 12 (G12V, G12D, G12C) and 13 (G13D). Exon 4: codon 146 (A146T; A146V)	Sanger Sequencing/ Pyrosequencing	FFPE pre-treatment biopsies	n = 57, Morocco	January 2012–October 2018	na	CRT ($n = 39$) or RT ($n = 18$) + surgery	5-FU	na	RFS (distant or local)	Carrying the KRAS exon 4 codon 146 mutations was associated with increased risk of developing metastasis ($p = 0.016$) and metastasis and/or recurrence ($p = 0.019$) by univariate analysis.	El Otmani et al. [49]
Exons 2, 3 and 4	EXPERT-C: Microarray INFINITI Platform (exons 2,3), Sanger Sequencing (exon 4). EXPERT: NGS	FFPE pre-treatment biopsies and/or post-treatment surgical samples	n = 210 (EXPERT-C trial: $n = 149$ and EXPERT trial: $n = 61$), Europe	2001–2008	I-II-III	CT followed by CRT and surgery	**CT**: CAPOX (+CTX for $n = 83$), **CRT**: CAPE (+CTX for $n = 78$)	EXPERT-C: CAPOX (+CTX for $n = 60$), EXPERT: CAPE.	5-yrs PFS	No significant association.	Sclafani et al. [50]
Codons 12–13	Sanger Sequencing/ Pyrosequencing/PCR assay/NGS	na	n = 3674 (**Meta-analysis: 16 studies**)	1988–2015 (Dec 2020 last literature research)	I-II-III-IV	CRT + surgery	Heterogeneous (5-FU/CAPE ± IRI ± CTX ± OXA)	na	DFS	Significant correlation between KRAS mutation and poor DFS (OR:1.55, 95% CI: 1.19–2.02)	Peng et al. [51]
G12D, G12A, G12V, G12S – G13D – T47P – G115E – A146T	Targeted DNA sequencing (72 cancer driving genes) and direct PCR sequencing	Frozen and FFPE pre-treatment biopsies	n = 76, Argentina	November 2015–September 2018	I-II-III	CRT ($n = 25$) or intensified CRT ($n = 36$) + surgery. Direct surgery ($n = 15$)	CAPE Intensified regimen: CAPOX + CAPE	Yes, ($n = 7$) with OXA.	Local RFS	KRAS mutational status was associated with higher risk of local recurrence (multivariate HR: 9.68; $p = 0.049$; long-rank $p = 0.0283$).	Iseas et al. [28]
G12D, G12A, G12V, G12S – G13D – T47P – G115E – A146T	Targeted DNA sequencing (72 cancer driving genes) and direct PCR sequencing	Frozen and FFPE pre-treatment biopsies	n = 76, Argentina	November 2015–Septebmer 2018	I-II-III	CRT ($n = 25$) or intensified CRT ($n = 36$) + surgery. Direct surgery ($n = 15$)	CAPE Intensified regimen: CAPOX + CAPE	Yes, ($n = 7$) with OXA.	RFS	KRAS mutational status was associated with worse RFS (univariate HR: 3.56; long-rank $p = 0.0061$).	Iseas et al. [28]
G12D, G12A, G12V, G12S – G13D – T47P – G115E – A146T	Targeted DNA sequencing (72 cancer driving genes) and direct PCR sequencing	Frozen and FFPE pre-treatment biopsies	n = 76, Argentina	November 2015–September 2018	I-II-III	CRT ($n = 25$) or intensified CRT ($n = 36$) + surgery. Direct surgery ($n = 15$)	CAPE Intensified regimen: CAPOX + CAPE	Yes, ($n = 7$) with OXA.	2 yrs – DFS	KRAS mutational status was associated with shorter DFS (multivariate HR: 2.55; $p = 0.039$; long-rank $p = 0.0113$).	Iseas et al. [28]

(*Continued*)

Table 1. (Continued).

Mutations or markers tested	Analytical method	Tumor Tissue Source	Study Population (n °/Country)	Enrollment Interval	Stage	Therapy strategy	CT agents*	Adjuvant Therapy*	Clinical End-point	Main findings	Ref
KRAS	n.a	Fresh frozen FFPE tumor tissue (timing not specified)	n = 77, Europe (Spain)	March 2007–August 2017	II – III	CT followed by surgery	Continuous infusion of 5-FU (*n* = 52)/ CAPE (*n* = 24)/ FOLFOX-6 (*n* = 1).	Yes, with 5-FU or CAPE as monotherapy or with OXA.	5 yrs – PFS	KRAS mutational status was associated with poorer PFS (MUT 36.8% vs WT 68.4%; *p* = 0.005).	Martìn-Carnicero et al. [52]
c) NRAS											
Exon 2 codon 12 (G12D); exon 3 codon 61 (Q61L)	Sanger Sequencing/ Pyrosequencing	FFPE pre-treatment biopsies	n = 57, Morocco	January 2012–October 2018	na	CRT (*n* = 39) or RT (*n* = 18) + surgery	5-FU	na	RFS (distant or local)	No significant association.	El Otmani et al. [49]
Exons 2, 3, 4	EXPERT-C: Microarray INFINITI Platform (exons 2,3), Sanger Sequencing (exon 4). EXPERT: NGS	FFPE pre-treatment biopsies and/ or post-treatment surgical samples	n = 210 (EXPERT-C trial: *n* = 149 and EXPERT trial: *n* = 61), Europe	2001–2008	I-II-III	CT followed by CRT and surgery	**CT**: CAPOX (+CTX for *n* = 83), **CRT**: CAPE (+CTX for *n* = 78)	EXPERT-C: CAPOX (+CTX for *n* = 60), EXPERT: CAPE.	5-yrs PFS	No significant association.	Sclafani et al. [50]
d) BRAF											
V600E	Pyrosequencing	FFPE pre- or post-treatment biopsies	n = 64, USA	na	II-III-IV	CRT + surgery	5-FU	na	local/distant recurrences	All three pts with BRAF mutation did not show local or distant recurrences (no recurrence: 62% WT vs 100% MUT)	Davies et al. [38]
Exon 15 (e.g. V600E)	Sanger Sequencing	FFPE pre-treatment biopsies	n = 98, Europe (France)	May 2006–September 2009	I-II-III	CRT + surgery	5-FU + LV or CAPE	na	local/distant recurrences	No significant association.	Derbel et al. [43]
V660E, D594G (exons 6, 11, 13, 14, 15 and 18)	NGS Illumina Miseq Platform + Sanger for variant confirmation	FFPE pre (74)- and post-treatment (9) biopsies	n = 74, China	January 2013–June 2016	II – III	nCRT + surgery	XELOX and mFOLFOX-6 accompanied by CAPE or 5-FU during RT	na	PFS	BRAF-mutated LARCs had shorter PFS (*p* = 0.045).	Jiang et al. [53]
Exon 15 (detected: V660E and D594G)	EXPERT-C: Microarray INFINITI Platform. EXPERT: NGS	FFPE pre-treatment biopsies and/ or post-treatment resection samples	n = 210 (EXPERT-C trial: *n* = 149 and EXPERT trial: *n* = 61), Europe	2001–2008	I-II-III	CT followed by CRT and surgery	**CT**: CAPOX (+CTX for *n* = 83), **CRT**: CAPE (+CTX for *n* = 78)	EXPERT-C: CAPOX (+CTX for *n* = 60), EXPERT: CAPE.	5-yrs PFS	No significant association.	Sclafani et al. [50]
e) PIK3CA											

(*Continued*)

Table 1. (Continued).

Mutations or markers tested	Analytical method	Tumor Tissue Source	Study Population (n °/Country)	Enrollment Interval	Stage	Therapy strategy	CT agents*	Adjuvant Therapy*	Clinical End-point	Main findings	Ref
Exon 9 (E542K, E545K, Q546K) and Exon 20 (Y1021C, T1025A, H1047R, T1052K)	Direct Sequencing	Fresh-frozen tumor post-treatment samples	n = 240, Europe (Netherlands)	January 1996–December 1999	I-II-III	Surgery (Total Mesorectal Excision)	na	na	Distant recurrence, 5 yrs risk distant metastasis.	No significant association. **Subgroup analysis:** pts with E545K PIK3CA mutation were associated with a poorer prognosis for distant recurrence (HR: 3.7; $p = 0.006$) than the remaining pts in multivariate analysis.	He et al. [54]
Exon 9 (E542K, E545K, Q546K) and Exon 20 (Y1021C, T1025A, H1047R, T1052K)	Direct Sequencing	Fresh-frozen tumor post-treatment samples	n = 240, Europe (Netherlands)	Januray 1996–December 1999	I-II-III	Surgery (Total Mesorectal Excision)	na	na	Local recurrence, 5 yrs risk local recurrence	PIK3CA mutations were associated with increased 5-yrs risk of local recurrence compared with the PIK3CA WT tumor (27.8% MUT vs 9.4% WT; $p = 0.008$). **Subgroup analysis:** **1)** higher local recurrence risk in subgroup of pts not receiving adjuvant RT (5-year risks, 26.7% versus 6.4%; $p = 0.004$); **2)** E545K PIK3CA mutation was associated with a decreased local recurrence (HR: 11.6; $p < 0.001$) compared to the remainders by multivariate analysis.	He et al. [54]
Exon 9 (E542K, E545K, Q546K) and Exon 20 (Y1021C, T1025A, H1047R, T1052K)	Direct Sequencing	Fresh-frozen tumor post-treatment samples	n = 240, Europe (Netherlands)	January 1996–December 1999	I-II-III	Surgery (Total Mesorectal Excision)	na	na	Overall recurrence	No significant association.	He et al. [54]
Exon 9 and 20	Sanger Sequencing	FFPE pre-treatment biopsies	n = 98, Europe (France)	May 2006–September 2009	I-II-III	CRT + surgery	5-FU + LV or CAPE	na	local/distant recurrences	No significant association.	Derbel et al. [43]

(*Continued*)

Table 1. (Continued).

Mutations or markers tested	Analytical method	Tumor Tissue Source	Study Population (n °/Country)	Enrollment Interval	Stage	Therapy strategy	CT agents*	Adjuvant Therapy*	Clinical End-point	Main findings	Ref
R88Q, H1047Y, R38H, C901F, M1043I, M1043I, N345K, C420R, P539R, E542K, E545K, Q546K, H701P, H1047R, H1047L	Sequenom MassARRAY system – OncoCarta Array	FFPE pre-treatment biopsies	n = 70, China	July 2006–June 2012	II-III	CRT + surgery	XELOX	XELOX	3 yrs – DSF	No significant association.	Peng et al. [33]
Exon 9 and 20	EXPERT-C: CE-SSCA and Sanger Sequencing. EXPERT: NGS	FFPE pre-treatment biopsies and/or post-treatment resection samples	n = 210 (EXPERT-C trial: *n* = 149 and EXPERT trial: *n* = 61), Europe	2001–2008	I-II-III	CT followed by CRT and surgery	**CT**: CAPOX (+CTX for *n* = 83), **CRT**: CAPE (+CTX for *n* = 78)	EXPERT-C: CAPOX (+CTX for *n* = 60), EXPERT: CAPE.	5-yrs PFS	No significant association.	Sclafani et al. [50]
Exon 9 and 20	PCR	FFPE post-treatment surgical samples	n = 128, Korea	January 2006–December 2011	na	nCRT	na	na	DFS	Pts with PIK3CA mutations had a significant shorter DFS than PIK3CA wild-type pts ($p = 0.006$).	Byun et al. [55]
f) TP53											
Exons 2 to 10	Direct sequencing	FFPE pre-treatment biopsies	n = 64, Europe (Austria)	1994–1998	I-II-III	Preoperative radiotherapy + surgery	na	Yes, for pts with node-positive tumors. (5-FU + LV)	Local and distant recurrence	No significant association.	Kandioler et al. [56]
Exons 2 to 10	Direct sequencing	FFPE pre-treatment biopsies	n = 64, Europe (Austria)	1994–1998	I-II-III	Preoperative radiotherapy + surgery	na	Yes, for pts with node-positive tumors. (5-FU + LV)	RFS	No significant association.	Kandioler et al. [56]
Exons 4, 5, 6, 7 and 8.	PCR – SSCP	FFPE pre and post (*n* = 12) treatment biopsies	n = 60, Australia	May 1991–Decemeber 1998	II-III	RT (*n* = 25)/CRT (*n* = 35)	5-FU and LV	na	Local recurrence	No significant association (overall). **Subgroup analysis:** mutations in exon 5 were associated with local recurrence in the chemoradiotherapy subgroup (2/6, 33%; $p = 0.03$). Mutations in exon 8 did not reach statistical significance ($p = 0.058$).	Saw et al. [57]

(*Continued*)

Table 1. (Continued).

Mutations or markers tested	Analytical method	Tumor Tissue Source	Study Population (n °/Country)	Enrollment Interval	Stage	Therapy strategy	CT agents*	Adjuvant Therapy*	Clinical End-point	Main findings	Ref
Exons 4–9 (EXPERT-C) Exons 4–11 (EXPERT)	EXPERT-C: CE-SSCA and Sanger Sequencing. EXPERT: NGS	FFPE pre-treatment biopsies and/or post-treatment resection samples	n = 210 (EXPERT-C trial: *n* = 149 and EXPERT trial: *n* = 61), Europe	2001–2008	I-II-III	CT followed by CRT and surgery	**CT**: CAPOX (+CTX for *n* = 83), **CRT**: CAPE (+CTX for *n* = 78)	EXPERT-C: CAPOX (+CTX for *n* = 60), EXPERT: CAPE.	5-yrs PFS	No significant association.	Sclafani et al. [50]
g) MSI											
BAT25, BAT26, NR21, NR24 and NR27 Considered MSI-H with ≥ 2 instable markers.	PCR assay, pentaplex of mononucleotide repeats (BAT25, BAT26, NR21, NR24 and NR27).	Paraffin-embedded pre-treatment biopsy samples	n = 316, China	January 1999–January 2007	II-III	RT + surgery + adjuvant CT	-	Yes, with OXA and 5-FU/CAPE.	5 yrs – DFS	No significant association. **Subgroup analysis:** pts with MSI-H tumors at the ypN0 stage had a significantly improved DFS compared to those with MSI-L and MSS tumors ($p = 0.040$)	Du et al. [58]
IHC staining: MLH1, MSH2, MSH6, PMS2. **PCR assay**: BAT-25, BAT-26, NR-21, NR-22, and NR-24 + hMLH1, hMSH2, and hMSH6 exons. Considered dMMR with ≥ 2 of instable markers.	IHC staining and PCR assay	FFPE post-treatment samples	n = 296, Europe (France)	January 2000–December 2016	III and favorable IV	CRT + surgery + adjuvant CT	FL-based chemotherapy (except 2 pts treated with short-course RT). Regimens: mFOLFOX4, CAPE, and 5FU either with LV.	Yes, depending on the pathologic stage. FL either alone or in association with OXA or IRI (FOLFOX *n* = 99 pts).	5 yrs – DFS	No significant association.	Meillan et al. [59]

(*Continued*)

Table 1. (Continued).

Mutations or markers tested	Analytical method	Tumor Tissue Source	Study Population (n °/Country)	Enrollment Interval	Stage	Therapy strategy	CT agents*	Adjuvant Therapy*	Clinical End-point	Main findings	Ref
IHC staining: MLH1, MSH2, MSH6, PMS2. **PCR assay**: BAT-25, BAT-26, NR-21, NR-22, and NR-24 + hMLH1, hMSH2, and hMSH6 exons. Considered dMMR with ≥ 2 of instable markers.	IHC staining and PCR assay	FFPE post-treatment samples	n = 296, Europe (France)	January 2000–December 2016	III and favorable IV	CRT + surgery	FL-based chemotherapy (except 2 pts treated with short-course RT). Regimens: mFOLFOX4, CAPE, and 5FU either with LV.	Yes, depending on the pathologic stage. FL either alone or in association with OXA or IRI (FOLFOX *n* = 99 pts).	Local Recurrence	No significant association.	Meillan et al. [59]
IHC staining: MLH1, MSH2, MSH6, PMS2. **PCR assay**: BAT-25, BAT-26, NR-21, NR-22, and NR-24 + hMLH1, hMSH2, and hMSH6 exons. Considered dMMR with ≥ 2 of instable markers.	IHC staining and PCR assay	FFPE post-treatment samples	n = 296, Europe (France)	January 2000–December 2016	III and favorable IV	CRT + surgery	FL-based chemotherapy (except 2 pts treated with short-course RT). Regimens: mFOLFOX4, CAPE, and 5FU either with LV.	Yes, depending on the pathologic stage. FL either alone or in association with OXA or IRI (FOLFOX *n* = 99 pts).	Metastatic Recurrence	No significant association.	Meillan et al. [59]
IHC staining: MLH1, MSH2, MSH6, PMS2. **PCR assay**: BAT-25, BAT-26, NR-21, NR-22, and NR-24 + hMLH1, hMSH2, and hMSH6 exons. Considered dMMR with ≥ 2 of instable markers.	IHC staining and PCR assay	FFPE post-treatment samples	n = 296, Europe (France)	Januray 2000–December 2016	III and favorable IV	CRT + surgery	FL-based chemotherapy (except 2 pts treated with short-course RT). Regimens: mFOLFOX4, CAPE, and 5FU either with LV.	Yes, depending on the pathologic stage. FL either alone or in association with OXA or IRI (FOLFOX *n* = 99 pts).	Recurrence	pMMR pts had higher recurrence (31.1% pMMR vs 8.7; $p = 0.0266$).	Meillan et al. [59]

(*Continued*)

Table 1. (Continued).

Mutations or markers tested	Analytical method	Tumor Tissue Source	Study Population (n °/Country)	Enrollment Interval	Stage	Therapy strategy	CT agents*	Adjuvant Therapy*	Clinical End-point	Main findings	Ref
IHC staining: MLH1, MSH2, MSH6, PMS2. **PCR assay**: BAT-25, BAT-26, NR-21, NR-22, and NR-24 + hMLH1, hMSH2, and hMSH6 exons. Considered dMMR with ≥ 2 of instable markers.	IHC staining and PCR assay	FFPE post-treatment samples	n = 296, Europe (France)	January 2000–December 2016	III and favorable IV	CRT + surgery + adjuvant CT	FL-based chemotherapy (except 2 pts treated with short-course RT). Regimens: mFOLFOX4, CAPE, and 5FU either with LV.	Yes, depending on the pathologic stage. FL either alone or in association with OXA or IRI (FOLFOX *n* = 99 pts).	5 yrs – RFS	RFS differed significantly between the pMMR and dMMR ($p = 0.0463$).	Meillan et al. [59]
MLH1, MSH2, MSH6, PMS2 Considered dMMR with loss of expression of at least 1 MMR proteins.	IHC	FFPE pre-treatment biopsies	n = 57, Morocco	January 2012–October 2018	na	CRT (*n* = 39) or RT (*n* = 18) + surgery	5-FU	na	RFS (distant or local)	No significant association.	El Otmani et al. [49]
BAT25, BAT26, NR21, NR24 and MONO27. Considered MSI-H with ≥ 2 instable markers.	PCR assay, pentaplex of mononucleotide markers (BAT25, BAT26, NR21, NR24 and MONO27).	Paraffin-embedded post-treatment samples	n = 549, Korea	January 2004–August 2015	na	Neoadjuvant CRT + surgery	5-FU or CAPE	na	5 yrs – DFS	No significant association.	Lee et al. [60]
IHC staining: MLH1, MSH2, MSH6, PMS2. **PCR assay**: BAT-25, BAT-26, NR-21, NR-24, MONO-27 (PCR assay. Considered MSI-H with ≥ 2 instable markers.	IHC staining and PCR assay with a panel for coamplification of five mononucleotide markers	FFPE pre and post treatment biopsy samples	n = 44, Japan	January 2017–October 2019	II-III	CRT + nivolumab + surgery	CAPE	Yes, some pts. (mFOLFOX6 or CAPOX)	Local, Distant and Overall recurrence	MSS pts: local recurrence 2/39, distant recurrence 4/39, overall recurrence 6/39. MSI-H pts: no local or distant recurrence.	Bando et al. [61]

(*Continued*)

Table 1. (Continued).

Mutations or markers tested	Analytical method	Tumor Tissue Source	Study Population (n °/Country)	Enrollment Interval	Stage	Therapy strategy	CT agents*	Adjuvant Therapy*	Clinical End-point	Main findings	Ref
MLH1, PMS2, MSH2 and MSH6. Considered dMMR with loss of expression of at least 1 MMR proteins.	PCR or immunohistochemistry (IHC).	na	n = 400, from 133 institutions across 40 countries.	2000–2020	I-II ($n = 97$) or III ($N = 208$) or unknown ($n = 95$).	CRT + surgery + adjuvant	na	Yes, for 249 pts	DFS (1, 3 and 5 yrs)	**MSI group**: DFS rate was 98, 90, and 87% at 1, 3, and 5 years, respectively. **MSS group**: DFS 89, 72, and 66% at 1, 3, and 5 years, respectively.	REACCT Collaborative, [62]
MLH1, PMS2, MSH2 and MSH6. Considered dMMR with loss of expression of at least 1 MMR proteins.	PCR or immunohistochemistry (IHC).	na	n = 400, from 133 institutions across 40 countries.	2000–2020	I-II ($n = 97$) or III ($N = 208$) or unknown ($n = 95$).	CRT + surgery	na	Yes, for 249 pts	Local recurrence	0 pts in the MSI group developed locoregional disease recurrence compared with 24 pts (6.9%) in the MSS group ($p = 0.159$).	REACCT Collaborative, [62]
MLH1, PMS2, MSH2 and MSH6. Considered dMMR with loss of expression of at least 1 MMR proteins.	PCR or immunohistochemistry (IHC).	na	n = 400, from 133 institutions across 40 countries.	2000–2020	I-II ($n = 97$) or III ($N = 208$) or unknown ($n = 95$).	CRT + surgery	na	Yes, for 249 pts	Metastatic recurrence	5 pts (10%) with MSI developed metastatic disease compared with 72 (20.6%) in the MSS group ($p = 0.084$).	REACCT Collaborative, [62]
MLH1, PMS2, MSH2 and MSH6. Considered dMMR with loss of expression of at least 1 MMR proteins.	Immunohistochemistry (IHC) and PCR if the result of IHC was uncertain.	FFPE tumor samples	n = 854, China	January 2013–December 2018	II – III	nCRT + surgery or nCT (no RT) + surgery	Infusional 5-FU or mFOLFOX6 or mFOLFOXIRI	n = 122 FL-based; $n =$ 614 OXA-based; $n =$ 118 no treatment.	DFS	dMMR status was associated with longer DFS (HR: 0.38; $p = 0.013$). 3-yrs DFS 93.2% dMMR vs 73.9% pMMR. **Subgroup analysis:** pts with ypStage II/III dMMR status had longer DFS compared to ypStage II/III pMMR (HR: 0.38; $p =$ 0.020).	Wu et al. [63]

(*Continued*)

Table 1. (Continued).

Mutations or markers tested	Analytical method	Tumor Tissue Source	Study Population (n °/Country)	Enrollment Interval	Stage	Therapy strategy	CT agents*	Adjuvant Therapy*	Clinical End-point	Main findings	Ref
MLH1, PMS2, MSH2 and MSH6. Considered dMMR with loss of expression of at least 1 MMR proteins.	Immunohistochemistry (IHC) and PCR if the result of IHC was uncertain.	FFPE tumor samples	n = 854, China	January 2013–December 2018	II – III	nCRT + surgery or nCT (no RT) + surgery	Infusional 5-FU or mFOLFOX6 or mFOLFOXIRI	n = 122 FL-based; *n* = 614 OXA-based; *n* = 118 no treatment.	LRFS	No significant association.	Wu et al. [63]
IHC staining: MLH1, MSH2, MSH6, PMS2. **PCR assay**: BAT-25, BAT-26, NR-21, NR-24, NR-27. Considered MSI with ≥ 2 instable markers.	Immunohistochemistry (IHC) and PCR analysis	FFPE pre- and post-treatment samples	n = 16.526, from different countries (**Meta-analysis: 22 studies**)	January 2022 (last literature research)	I -IV	Heterogeneous	Heterogeneous	Heterogeneous	DFS	No significant association.	Swets et al. [64]
MLH1, PMS2, MSH6, MSH2, and EPCAM Considered dMMR with loss of expression of at least 1 MMR proteins.	Immunohistochemistry (IHC)	Surgical samples	n = 318, (exploratory cohort *n* = 160, validation cohort *n* = 158), Europe (Italy)	na	II – III	CRT + surgery + adjuvant CT	CAPE	Yes, for 63 pts in the exploratory cohort (information n.a. for the validation cohort).	DFS	Exploratory cohort: pMMR showed a higher DFS compared to dMMR (NR vs 14 months, $p = 0.003$). Validation cohort: pMMR showed a higher DFS compared to dMMR (NR vs 17 months, $p = 0.02$).	Pretta et al. [65]
h) SMAD4											
R361H, D355G, D351N, D351G, G365D, R445*, D537Y, R361C, R361H (exons 9, 10, 11 and 12)	NGS Illumina Miseq Platform + Sanger for variant confirmation	FFPE pre (74)- and post-treatment (9) biopsies	n = 74, China	January 2013–Junuary 2016	II – III	nCRT + surgery	XELOX and mFOLFOX-6 accompanied by CAPE or 5-FU during RT	na	PFS	SMAD4-mutated LARCs had shorter PFS ($p = 0.008$).	Jiang et al. [53]
i) MULTIPLE											

(*Continued*)

Table 1. (Continued).

Mutations or markers tested	Analytical method	Tumor Tissue Source	Study Population (n °/Country)	Enrollment Interval	Stage	Therapy strategy	CT agents*	Adjuvant Therapy*	Clinical End-point	Main findings	Ref
PIK3CA: exon 9 (E542K, E545K, Q546K) and exon 20 (Y1021C, T1025A, H1047R, T1052K). KRAS: exon 1; codon 12 and 13 (G12S, G12R, G12C, G12D, G12A, G12V, G13C, G13D, G13R, V14I).	Direct Sequencing	Fresh-frozen tumor post-treatment samples	n = 240, Europe (Netherlands)	January 1996–December 1999	I-II-III	Surgery (Total Mesorectal Excision)	na	na	Local recurrence (5 yrs risk) and distant metastasis	Ten pts with concomitant mutations in PIK3CA and KRAS showed a higher local recurrence rate than pts having no or single gene mutation (5-year risks, 33.3% vs 10%; $p = 0.012$). The concomitant mutations showed no correlation with distant metastases ($p = 0.868$).	He et al. [54]
RAS/BRAF (detailed mutation status: KRAS $n = 20$, NRAS $n = 2$, BRAF $n = 2$)	PCR analysis	Pre-treatment biopsy tissues	n = 57, Japan	January 2009–March 2016	II – III	NAC + surgery	XELOX ($n = 32$) or XELOX + BEV ($n = 21$) or mFOLFOX6 ($n = 4$)	na	3 yrs – RFS	The 3 yrs – RFS was better in WT that in the MT group (95% vs 59%, $p = 0.011$).	Oshiro et al. [66]
KRAS and TP53 whole exome	NGS	FFPE pre and post matched treatment samples	n = 17, USA	2010–2016	II – III	CRT + surgery	FL-based	FOLFOX	5 yrs – PFS	Pts with concurrent KRAS and TP53 mutations experienced reduced 5-yrs PFS compared with those without (38% vs 90%, log-rank $p = 0.04$).	Kamran et al. [67]
TP53 (exons 4–11) and KRAS/NRAS (exons 2–4)	EXPERT-C: CE-SSCA and Sanger Sequencing (exon 3), Sanger Sequencing (exons 2, 4). EXPERT: NGS	FFPE pre-treatment biopsies and/or post-treatment resection samples	n = 210 (EXPERT-C trial: $n = 149$ and EXPERT trial: $n = 61$), Europe	2001–2008	I-II-III	CT followed by CRT and surgery	**CT**: CAPOX (+CTX for $n = 83$), **CRT**: CAPE (+CTX for $n = 78$)	EXPERT-C: CAPOX (+CTX for $n = 60$), EXPERT: CAPE.	5-yrs PFS	Pts with TP53 and KRAS/NRAS mutated tumors had a worse 5-yrs PFS than those with TP53/KRAS/NRAS wild-type tumors (MUT: 54% vs WT:72%, $p = 0.02$)	Sclafani et al. [50]

Abbreviations: CE-SSCA, Capillary electrophoresis single-strand; CRC, colorectal cancer; CRT, chemoradiotherapy; CT, chemotherapy; DFS, disease-free survival; dMMR, deficient mismatch repair; FFPE, formalin-fixed paraffin Embedded; IHC, immunohistochemistry; LRFS, local recurrence-free survival; MSI, microsatellite instability/microsatellite instable; MSI-H, high microsatellite instable; MSI-L, low microsatellite instable; MSS, microsatellite stable MUT, mutated; NAC, neoadjuvant chemotherapy; nCRT, neoadjuvant chemoradiotherapy; NGS, next-generation sequencing; OS, overall survival; PCR, polymerase chain reaction; pMMR, proficient mismatch repair; PFS, Progression- free survival; pts, patients; RFA, radiofrequency ablation; RFS, relapse-free survival; RT, radiotherapy; SSCP,single-strand conformation polymorphism; TNT, total neoadjuvant treatment; WT, wild-type.

***CT agents and Regimens**: 5-FU: 5-fluorouracil; CAPE: capecitabine; CAPOX/XELOX: capecitabine and oxaliplatin; CTX: cetuximab; FL, fluoropyrimidines; FOLFOX: 5-FU, leucovorin and oxaliplatin; mFOLFOX-4/FOLFOX-6/mFOLFOX-6: oxaliplatin, 5-FU and leucovorin; IRI: irinotecan; LV, leucovorin; mFOLFOXIRI: 5-FU, leucovorin, oxaliplatin and irinotecan; OXA: oxaliplatin; XELOX + BEV: capecitabine, oxaliplatin and bevacizumab.

Table 2. Published works on the impact of somatic mutation in oncogenes and MSI status on survival in locally advanced rectal cancer (LARC) treated with preoperative radiation-based therapy.

Mutations or markers tested	Analytical method	Tumor Tissue Source	Study Population (n °/Country)	Enrollment Interval	Stage	Therapy strategy	CT agents*	Adjuvant Therapy*	Clinical End-point	Main findings	Ref
a) All RAS											
KRAS codons 12, 13, 61, 146, **NRAS** codons 12, 13, 61.	Pyrosequencing and NGS	FFPE pretreatment biopsies and surgical resection tumor tissues	n = 78, UK	April 2009–October 2011	II – III	CRT + surgery	CAPE, CTX and IRI	FL ($n = 23$) or FL +OXA ($n = 28$) and $n = 3$ unspecified regimen	OS	Improvement in OS for WT compared to RAS mutated cancers (HR 0.32 (95%CI: 0.09 to 1.14)).	Gollins et al. [32]
KRAS (G12V, G12A, G12D, G12C, G12S, G12R, G12F, G13V, G13D, A59T, Q61E, Q61K, Q61L, Q61R, Q61P, Q61H, Q61H); **NRAS** (G12V, G12A, G12D, G12C, G12R, G12S, G13V, G13A, G13D, G13C, G13R, G13S, A18T, Q61L, Q61R, Q61P, Q61H, Q61E, Q61K)	Sequenom MassARRAY system – OncoCarta Array	FFPE pre-treatment biopsies	n = 70, China	July 2006–June 2012	II-III	CRT + surgery	XELOX	XELOX	3 yrs – OS	RAS mutations were associated with lower OS-3 yrs rate than RAS WT (89.7% vs 94.4%, $p = 0.036$).	Peng et al. [33]
KRAS (codons 12, 13, 59, 61, 117 and 146). **NRAS** (codons 12, 13, 58, 59, 61, 117 and 146).	Pyrosequencing	Pre- or post-treatment specimens (formalin fixed and kerosene included)	n = 39, Europe (Italy)	May 2011–June 2017	II – III	nCRT + surgery	5-FU or CAPE	Yes, $n = 16$. XELOX or FOLFOX.	5 yrs – OS	The 5-year OS was significantly lower in RAS-mutated pts than in RAS wild-type pts ($p = 0.0022$)	Orlandi et al. [35]
b) KRAS											
Codons 12–13 and 61: G12D, G12S, G13D, G12A and Q61H	PCR, RFLP and SSCP tecniques	Frozen post-treatment biopsy	n = 37, Mexico	May 1993–November 1995	I-II-III-IV	CRT + surgery	5-FU	na	5 yrs – OS	OS in pts with mutated KRAS was 100%, whereas in WT pts it was 59% ($p = 0.03$).	Luna-Pérez et al. [68]
Codons 12–13	Sanger Sequencing	FFPE pre-treatment biopsies	n = 146, Europe (Italy)	May 1998–October 2005	II-III-IV	CRT + surgery	5-FU ($n = 98$), 5-FU+OXA ($n = 34$), CAPE ($n = 14$)	na	5 yrs – OS	No significant association.	Bengala et al. [37]
Codons 12-13-61	Pyrosequencing	FFPE pre- or post-treatment biopsies	n = 67, USA	na	II-III-IV	CRT + surgery	5-FU	na	OS	No significant association.	Davies et al. [38]

(*Continued*)

Table 2. (Continued).

Mutations or markers tested	Analytical method	Tumor Tissue Source	Study Population (n °/Country)	Enrollment Interval	Stage	Therapy strategy	CT agents*	Adjuvant Therapy*	Clinical End-point	Main findings	Ref
Codons 12–13	Pyrosequencing	FFPE pre-treatment biopsies	n = 63, China	September 2007–March 2008	II-III	CRT + surgery	CAPE + CTX	Yes, some pts. Unspecified regimen.	3 yrs – OS	No significant association.	Sun et al. [41]
Codons 12-13-21-60-61-146	Pyrosequencing and direct sequencing	FFPE pre- and post-treatment samples	n = 696 (**Meta-analysis: 8 studies**)	1995–2011 (October 2012 last literature research)	II-III-IV	CRT + surgery	Heterogeneous (CAPE/5-FU ± CTX ± OXA ± IRI)	na	Survival	No significant association.	Clancy et al. [69]
Exon 2 (e.g. codon 12: G12D, G12V, G12S, G12R, G12C; codon 13: G13D) and exon 3	Sanger Sequencing	FFPE pre-treatment biopsies	n = 98, Europe (France)	May 2006–September 2009	I-II-III	CRT + surgery	5-FU + LV or CAPE	na	OS	No significant association.	Derbel et al. [43]
Exon 1: codons 12–13, exon 2	Sanger Sequencing	FFPE pre- or post-treatment biopsies	n = 48, Germany	February 2005–March 2006	II-III-IV	CRT + surgery	CTX-CAPE-OXA	No ($n = 17$), 5-FU/LV or CAPE ($n = 15$), 5-FU/LV or CAPE+OXA ($n = 20$), RFA ($n = 1$).	OS	No significant association.	Fokas et al. [44]
Codons 12 (G12A, G12C, G12D, G12S, and G12V) – 13 (G13D)	PCR assay	FFPE pre-treatment biopsies	n = 100, Korea	December 2008–September 2013	II-III	CRT + surgery	5-FU + LV	5-FU	3 yrs – OS	No significant association.	Lee et al. [45]
KRAS (codons 12 (G12D, G12V, G12A, G12C, G12S) –13 (G13D))	Sanger Sequencing	FFPE pre-treatment biopsies	n = 63, Europe (Serbia)	2006–2010	II-III	CRT + surgery	5-FU + LV	na	OS	No significant association. **Subgroup analysis:** pts with concurrent KRAS mutation and high VEGF expression showed significantly shorter OS compared to the other ($p = 0.001$).	Krajnović et al. [47]

(*Continued*)

Table 2. (Continued).

Mutations or markers tested	Analytical method	Tumor Tissue Source	Study Population (n °/Country)	Enrollment Interval	Stage	Therapy strategy	CT agents*	Adjuvant Therapy*	Clinical End-point	Main findings	Ref
G12V, G12A, G12D, G12C, G12S, G12R, G12F, G13V, G13D, A59T, Q61E, Q61K, Q61L, Q61R, Q61P, Q61H, Q61H	Sequenom MassARRAY system – OncoCarta Array	FFPE pre-treatment biopsies	n = 70 China	July 2006–June 2012	II-III	CRT + surgery	XELOX	XELOX	3 yrs – OS	KRAS mutations were associated with lower OS-3 yrs rate than KRAS WT (88% vs 95.4%, p = 0.020). **Subgroup analysis:** pts with the KRAS subtype G12D (GGT > GAT) (7/70; 10%) showed poorer survival than all others (71.4 vs 95.1%, p = 0.028).	Peng et al. [33]
Codons 12–13	PCR assay	FFPE biopsies	n = 60, Egypt	March 2014–December 2015	I-II-III	na	na	na	5 yrs – OS	Cancer pts carrying KRAS mutation had a reduced 5 yrs-OS compared to WT KRAS (20.8% vs 69.4%, p = 0.001).	Bahnassy et al. [48]
Exon 2 codon 12 (G12V, G12D, G12C) and 13 (G13D); exon 4 codon 146 (A146T; A146V)	Sanger Sequencing/ Pyrosequencing	FFPE pre-treatment biopsies	n = 57 Morocco	January 2012–October 2018	na	CRT (n = 39) or RT (n = 18) + surgery	5-FU	na	OS	No significant association.	El Otmani et al. [49]
Exons 2, 3, 4	EXPERT-C: Microarray INFINITI Platform (exons 2,3), Sanger Sequencing (exon 4). EXPERT: NGS	FFPE pre-treatment biopsies and/or post-treatment resection samples	n = 210 (EXPERT-C trial: n = 149 and EXPERT trial: n = 61), Europe	2001–2008	I-II-III	CT followed by CRT and surgery	**CT**: CAPOX (+CTX for n = 83), **CRT**: CAPE (+CTX for n = 78)	EXPERT-C: CAPOX (+CTX for n = 60), EXPERT: CAPE.	5-yrs OS	No significant association.	Sclafani et al. [50]
KRAS	na	na	n = 1886 USA (84% Caucasian)	2010–2015	II-III	CRT/intensified CRT + surgery	Mono-agent or multi-agents **	Yes (in a subgroup of pts)	OS	KRAS mutation was associated with worse OS (p = 0.02).	Zhou et al. [70]
KRAS (mainly codons 12–13)	Sanger Sequencing/ Pyrosequencing/PCR assay/NGS	na	n = 3674 (**Meta-analysis: 16 studies**)	1988–2015 (December 2020 last literature research)	I-II-III-IV	CRT + surgery	Heterogeneous (5-FU/CAPE ± IRI ± CTX ± OXA)	na	OS	Significant correlation between KRAS mutation and poor OS (OR:1.33, 95%CI: 1.13–1.56)	Peng et al. [51]

(*Continued*)

Table 2. (Continued).

Mutations or markers tested	Analytical method	Tumor Tissue Source	Study Population (n °/Country)	Enrollment Interval	Stage	Therapy strategy	CT agents*	Adjuvant Therapy*	Clinical End-point	Main findings	Ref
KRAS	na	na	n = 784, USA	2004–2015	II-III	CRT + surgery	na	na	3 yrs and 5 yrs – OS	KRAS mutation was associated with poorer survival. 3 yr OS: MUT 79,9% vs WT 83,6%; 5 yr OS: MUT 56,7% vs WT 61,9%.	Asawa et al. [71]
G12D, G12A, G12V, G12S – G13D – T47P – G115E – A146T	Targeted DNA sequencing (72 cancer driving genes) and direct PCR sequencing	Frozen and FFPE pre-treatment biopsies	n = 76, Argentina	November 2015–September 2018	I-II-III	CRT ($n = 25$) or intensified CRT ($n = 36$) + surgery. Direct surgery ($n = 15$)	CAPE Intensified regimen: CAPOX + CAPE	Yes, ($n = 7$) with OXA.	2 yrs – OS	KRAS mutational status was associated with shorter OS (univariate HR: 3.50; $p = 0.037$; long-rank $p = 0.026$).	Iseas et al. [28]
G12D, G12A, G12V, G12S – G13D – T47P – G115E – A146T	Targeted DNA sequencing (72 cancer driving genes) and direct PCR sequencing	Frozen and FFPE pre-treatment biopsies	n = 76, Argentina	November 2015–September 2018	I-II-III	CRT ($n = 25$) or intensified CRT ($n = 36$) + surgery. Direct surgery ($n = 15$)	CAPE Intensified regimen: CAPOX + CAPE	Yes, ($n = 7$) with OXA.	Specific OS	KRAS mutational status was associated with shorter specific OS (univariate HR: 4.15; $p = 0.035$; long-rank $p = 0.022$).	Iseas et al. [28]
KRAS	n.a	Fresh frozen FFPE tumor tissues (timing not specified)	n = 77, Europe (Spain)	March 2007–August 2017	II – III	CT followed by surgery	Continuous infusion of 5-FU ($n = 52$)/ CAPE ($n = 24$)/ FOLFOX-6 ($n = 1$).	Yes, with 5-FU or CAPE as monotherapy or with OXA.	5 yrs – OS	KRAS mutational status was associated with poorer OS (MUT 47.4% vs WT 73.7%; $p = 0.022$).	Martìn-Carnicero et al. [52]
c) NRAS											
Exon 2 codon 12 (G12D); exon 3 codon 61 (Q61L)	Sanger Sequencing/ Pyrosequencing	FFPE pre-treatment biopsies	n = 57, Morocco	January 2012–October 2018	na	CRT ($n = 39$) or RT ($n = 18$) + surgery	5-FU	na	OS	No significant association	El Otmani et al. [49]
Exons 2, 3, 4	EXPERT-C: CE-SSCA and Sanger Sequencing (exon 3), Sanger Sequencing (exons 2, 4). EXPERT: NGS	FFPE pre-treatment biopsies and/ or post-treatment resection samples	n = 210 (EXPERT-C trial: $n = 149$ and EXPERT trial: $n = 61$), Europe	2001–2008	I-II-III	CT followed by CRT and surgery	**CT**: CAPOX (+CTX for $n = 83$), **CRT**: CAPE (+CTX for $n = 78$)	EXPERT-C: CAPOX (+CTX for $n = 60$), EXPERT: CAPE.	5-yrs OS	No significant association.	Sclafani et al. [50]
d) BRAF											
V600E	Pyrosequencing	FFPE pre- or post-treatment biopsies	n = 64, USA	na	II-III-IV	CRT + surgery	5-FU	na	OS	No significant association	Davies et al. [38]

(*Continued*)

Table 2. (Continued).

Mutations or markers tested	Analytical method	Tumor Tissue Source	Study Population (n °/Country)	Enrollment Interval	Stage	Therapy strategy	CT agents*	Adjuvant Therapy*	Clinical End-point	Main findings	Ref
Exon 15 (e.g. V600E)	Sanger Sequencing	FFPE pre-treatment biopsies	n = 98, Europe (France)	May 2006–September 2009	I-II-III	CRT + surgery	5-FU + LV or CAPE	na	OS	No significant association	Derbel et al. [43]
V660E, D594G (exons 6, 11, 13, 14, 15 and 18)	NGS Illumina Miseq Platform + Sanger for variant confirmation	FFPE pre (74)- and post-treatment (9) biopsy	n = 74, China	January 2013–June 2016	II – III	nCRT + surgery	XELOX and mFOLFOX-6 accompanied by CAPE or 5-FU during RT	na	OS	BRAF-mutated LARC pts had shorter OS (p = 0.000).	Jiang et al. [53]
Exon 15 (detected: V660E and D594G)	EXPERT-C: Microarray INFINITI Platform. EXPERT: NGS	FFPE pre-treatment biopsies and/or post-treatment resection samples	n = 210 (EXPERT-C trial: n = 149 and EXPERT trial: n = 61), Europe	2001–2008	I-II-III	CT followed by CRT and surgery	**CT**: CAPOX (+CTX for n = 83), **CRT**: CAPE (+CTX for n = 78)	EXPERT-C: CAPOX (+CTX for n = 60), EXPERT: CAPE.	5-yrs OS	No significant overall association. **Subgroup analysis:** in the group of pts who did not receive CTX, BRAF mutation was associated with worse 5-yrs OS (MUT 20% vs WT 73%, p = 0.03).	Sclafani et al. [50]
e) PIK3CA											
Exon 9 (E542K, E545K, Q546K) and Exon 20 (Y1021C, T1025A, H1047R, T1052K)	Direct Sequencing	Fresh-frozen tumor post-treatment samples	n = 240, Europe (Netherlands)	January 1996–December 1999	I-II-III	Surgery (Total Mesorectal Excision)	na	na	OS	No significant association.	He et al. [54]
Exon 9 and 20	Sanger Sequencing	FFPE pre-treatment biopsies	n = 98, Europe (France)	May 2006–September 2009	I-II-III	CRT + surgery	5-FU + LV or CAPE	na	OS	No significant association.	Derbel et al. [43]
R88Q, H1047Y, R38H, C901F, M1043I, M1043I, N345K, C420R, P539R, E542K, E545K, Q546K, H701P, H1047R, H1047L	Sequenom MassARRAY system – OncoCarta Array	FFPE pre-treatment biopsy	n = 70, China	July 2006–June 2012	II-III	CRT + surgery	XELOX	XELOX	3 yrs – OS	No significant association.	Peng et al. [33]

(*Continued*)

Table 2. (Continued).

Mutations or markers tested	Analytical method	Tumor Tissue Source	Study Population (n °/Country)	Enrollment Interval	Stage	Therapy strategy	CT agents*	Adjuvant Therapy*	Clinical End-point	Main findings	Ref
Exon 9 and 20	EXPERT-C: CE-SSCA and Sanger Sequencing. EXPERT: NGS	FFPE pre-treatment biopsies and/or post-treatment resection samples	n = 210 (EXPERT-C trial: *n* = 149 and EXPERT trial: *n* = 61), Europe	2001–2008	I-II-III	CT followed by CRT and surgery	**CT**: CAPOX (+CTX for *n* = 83), **CRT**: CAPE (+CTX for *n* = 78)	EXPERT-C: CAPOX (+CTX for *n* = 60), EXPERT: CAPE.	5-yrs OS	No significant association.	Sclafani et al. [50]
Exon 9 and 20	PCR	FFPE post-treatment surgical samples	n = 128, Korea	January 2006–December 2011	na	nCRT	na	na	CSS	Pts with PIK3CA mutations had a significant shorter CSS than PIK3CA wild-type pts ($p = 0.001$).	Byun et al. [55]
f) TP53											
Exons 2 to 10	Direct sequencing	FFPE pre-treatment biopsies	n = 64, Europe (Austria)	1994–1998	I-II-III	Preoperative RT + surgery	na	Yes, for pts with node-positive tumors. (5-FU + LV)	OS	Overall survival was diminished in pts with mutated p53 gene ($p = 0.049$).	Kandioler et al. [56]
Exons 4, 5, 6, 7 and 8.	PCR – SSCP	FFPE pre and post (*n* = 12) treatment biopsies	n = 60, Australia	May 1991–December 1998	II-III	RT (*n* = 25)/CRT (*n* = 35)	5-FU and LV	na	Cancer-specific survival	No significant association.	Saw et al. [57]
Exons 4–9 (EXPERT-C) Exons 4–11 (EXPERT)	EXPERT-C: CE-SSCA and Sanger Sequencing. EXPERT: NGS	FFPE pre-treatment biopsies and/or post-treatment resection samples	n = 210 (EXPERT-C trial: *n* = 149 and EXPERT trial: *n* = 61), Europe	2001–2008	I-II-III	CT followed by CRT and surgery	**CT**: CAPOX (+CTX for *n* = 83), **CRT**: CAPE (+CTX for *n* = 78)	EXPERT-C: CAPOX (+CTX for *n* = 60), EXPERT: CAPE.	5-yrs OS	No significant association.	Sclafani et al. [50]

(*Continued*)

Table 2. (Continued).

Mutations or markers tested	Analytical method	Tumor Tissue Source	Study Population (n °/Country)	Enrollment Interval	Stage	Therapy strategy	CT agents*	Adjuvant Therapy*	Clinical End-point	Main findings	Ref
g) MSI											
IHC staining: MLH1, MSH2, MSH6, PMS2 **PCR assay**: BAT25, BAT26, D2S123, D5S346 and D17S250 Considered MSI-H with ≥ 2 instable markers.	IHC and PCR assay	Post-treatment paraffin-embedded tumor tissues	n = 341, Turkey	January 2014–August 2018	II-III	CRT + surgery + adjuvant CT	5-FU or CAPE	FOLFOX	OS	No significant association.	Acar et al. [72]
IHC staining: MLH1, MSH2, MSH6, PMS2. **PCR assay**: BAT-25, BAT-26, NR-21, NR-22, and NR-24 + hMLH1, hMSH2, and hMSH6 exons. Considered dMMR with ≥ 2 of instable markers.	IHC staining and PCR assay	FFPE post-treatment samples	n = 296, Europe (France)	January 2000–December 2016	III and favorable IV	CRT + surgery + adjuvant CT	FL-based chemotherapy (except 2 pts treated with short-course RT). Regimens: mFOLFOX4, CAPE, and 5FU either with LV.	Yes, depending on the pathologic stage. FL either alone or in association with OXA or IRI (FOLFOX *n* = 99 pts).	5 yrs – OS	No significant association.	Meillan et al. [59]
MLH1, MSH2, MSH6, PMS2 Considered dMMR with loss of expression of at least 1 MMR proteins.	IHC	FFPE pre-treatment biopsies	n = 57, Morocco	January 2012–October 2018	na	CRT (*n* = 39) or RT (*n* = 18) + surgery	5-FU	na	OS	No significant association	El Otmani et al. [49]
na	na	na	n = 3902, USA	2010–2015	II-III	CRT + surgery	na	na	3 and 5 yrs OS	No significant association.	Hasan et al. [73]

(*Continued*)

Table 2. (Continued).

Mutations or markers tested	Analytical method	Tumor Tissue Source	Study Population (n °/Country)	Enrollment Interval	Stage	Therapy strategy	CT agents*	Adjuvant Therapy*	Clinical End-point	Main findings	Ref
BAT25, BAT26, NR21, NR24 and MONO27. Considered MSI-H with ≥ 2 instable markers.	PCR assay, pentaplex of mononucleotide markers (BAT25, BAT26, NR21, NR24 and MONO27).	Paraffin-embedded post-treatment samples	n = 549, Korea	January 2004–August 2015	na	Neoadjuvant CRT + surgery	5-FU or CAPE	na	5 yrs – OS	No significant association.	Lee et al. [60]
IHC staining: MLH1, MSH2, MSH6, PMS2. **PCR assay**: BAT-25, BAT-26, NR-21, NR-24, MONO-27 (PCR assay. Considered MSI-H with ≥ 2 instable markers.	IHC staining and PCR assay with a panel for co-amplification of five mononucleotide markers (NR-21, BAT-25, MONO-27, NR-24 and BAT-26)	FFPE pre and post treatment biopsy samples	n = 44, Japan	January 2017–October 2019	II-III	CRT + nivolumab + surgery + adjuvant CT	CAPE	Yes, some pts. (mFOLFOX6 or CAPOX)	OS	MSS pts: 1 death. MSI-H pts: no death.	Bando et al. [61]
MLH1, PMS2, MSH2 and MSH6. Considered dMMR with loss of expression of at least 1 MMR proteins.	PCR or immunohistochemistry (IHC).	na	n = 400, from 133 institutions across 40 countries.	2000–2020	I-II (*n* = 97) or III (*n* = 208) or unknown (*n* = 95).	CRT + surgery + adjuvant CT	na	Yes, for 249 pts	OS (1, 3 and 5 yrs)	**MSI group**: median OS was 58 months, with 1-, 3-, and 5-year OS rates of 100, 95, and 89% respectively. **MSS group**: median OS was 32 months, and 96, 90, and 84%.	REACCT Collaborative, [62]

(*Continued*)

Table 2. (Continued).

Mutations or markers tested	Analytical method	Tumor Tissue Source	Study Population (n °/Country)	Enrollment Interval	Stage	Therapy strategy	CT agents*	Adjuvant Therapy*	Clinical End-point	Main findings	Ref
IHC staining: MLH1, MSH2, MSH6, PMS2. **PCR assay**: BAT-25, BAT-26, NR-21, NR-24, NR-27. Considered MSI with ≥ 2 instable markers.	Immunohistochemistry (IHC) and PCR analysis	FFPE pre- and post-treatment samples	n = 16.526, from different countries (**Meta-analysis: 22 studies**)	January 2022 (last literature research)	I -IV	Heterogeneous	Heterogeneous	Heterogeneous	OS	No significant association.	Swets et al. [64]
h) SMAD4											
R361H, D355G, D351N, D351G, G365D, R445*, D537Y, R361C, R361H (exons 9, 10, 11 and 12	NGS Illumina Miseq Platform + Sanger for variant confirmation	FFPE pre (74)- and post-treatment (9) biopsies	n = 74, China	Januray 2013–June 2016	II – III	nCRT + surgery	XELOX and mFOLFOX-6 accompanied by CAPE or 5-FU during RT	na	OS	No significant association.	Jiang et al. [53]

Abbreviations: CE-SSCA, Capillary electrophoresis single-strand; CRT, chemoradiotherapy; CT, chemotherapy; dMMR, deficient mismatch repair; FFPE, formalin-fixed paraffin Embedded; IHC, immunohistochemistry; MSI, microsatellite instability/microsatellite instable; MSI-H, high microsatellite instable; MSI-L, low microsatellite instable; MSS, microsatellite stable MUT, mutated;nCRT, neoadjuvant chemoradiotherapy; NGS, next-generation sequencing; OS, overall survival; PCR, polymerase chain reaction; pMMR, proficient mismatch repair; pts, patients; RFA, radiofrequency ablation; RFLP, restriction fragment length polymorphism; RT, radiotherapy; SSCP,single-strand conformation polymorphism; TNT, total neoadjuvant treatment; WT, wild-type.

***CT agents and Regimens**: 5-FU: 5-fluorouracil; CAPE: capecitabine; CAPOX/XELOX: capecitabine and oxaliplatin; CTX: cetuximab; FL, fluoropyrimidines; FOLFOX: 5-FU, leucovorin and oxaliplatin; mFOLFOX-4/FOLFOX-6/mFOLFOX-6: oxaliplatin, 5-FU and leucovorin; IRI: irinotecan; LV, leucovorin; OXA: oxaliplatin.

****3 group**: (1) neoadjuvant multiagent chemoradiation or total neoadjuvant therapy, (2) neoadjuvant chemoradiation and multi-agent adjuvant chemotherapy, and (3) mono-agent neoadjuvant/adjuvant chemoradiation.

obtain a more homogeneous study population. Another point to note is that the works published before 2016 were based on *KRAS* mutational assays performed either in pre-treatment biopsies or in post-treatment biological samples. The molecular architecture of biological material that has already been exposed to chemoradiation therapy may have been affected by it and therefore may not reflect the basal mutational status of *KRAS*. In addition, in the studies prior to 2016, the preoperative chemoradiotherapy (CRT) regimen was more heterogeneous and included the use of CTX (5/11 studies, 45.5%) or CTX and IRI (2/11, 18.2%). The use of CTX may have introduced a bias into the analysis. Indeed, this monoclonal antibody against the epidermal growth factor receptor (EGFR) was indicated in a recent meta-analysis to alter the predictive value of *KRAS* status for the probability of achieving a pCR [31]. Notably, the negative impact of *KRAS* mutations on tumor response was observed only in LARC patients not treated with CTX, while the predictive value for *KRAS* was lost in patients treated with the biological agent. It is likely that the completely different molecular architecture of rectal cancer alters the interaction between CTX and *KRAS* mutations previously observed in colon cancer [31]. After 2016, the use of CTX was completely abolished in clinical practice, so studies published after that year did not include this drug in the nCRT regimen. The only exception was the paper by Sclafani et al. [50] in which the treatment schedule included CTX for a subset of patients. This is due to the fact that this study examined patients who had participated in two large phase II clinical trials between 2001 and 2008, when this biological agent was still part of the treatment setting.

A meta-analysis published in 2021 [51] was also found by literature search (Table 1(b)). It includes 16 papers published between 2010 and 2020, 11 of which were also included in our review. Considering the above-mentioned bias introduced by the use of CTX, it is important to note that only two of the papers included in the meta-analysis comprehended the anti-EGFR agent in the treatment regimen. This meta-analysis further confirmed that the presence of *KRAS* gene mutations was associated with reduced DFS (OR:1.55, 95%CI:1.19–2.02) [51].

It should be noted that examining the entire study population may overlook some associations that can be uncovered by performing specific subgroup analysis. Grouping patients by clinicopathological characteristics (e.g. tumor location [47] or molecular characteristics (e.g. specific *KRAS* codon mutations [49]) could potentially reveal an overlooked association [31]. For example, Krajnovic et al. [47] reported that patients with a tumor at 7 cm from the anal verge and *KRAS* mutations had more local and distant recurrences than patients with the same tumor location and wild-type *KRAS* (wild-type vs. mutated: 20% vs. 59%, $p = 0.036$). Similarly, a sub-analysis by El Otmani et al. [49] showed an association between the presence of mutations in codon 146 of the *KRAS* gene and an increased risk of recurrence ($p = 0.019$). In addition, a polygenic risk score that takes into account the interplay of several signaling pathways could probably better capture the phenotype of tumor recurrence. In the work by Krajnovic et al. [47], for example, it was shown that a mutation in the *KRAS* gene and simultaneous high expression of vascular endothelial growth factor (VEGF) entails an increased risk of local and distant recurrence during disease development (wild type vs. mutated *KRAS*/high VEGF, occurrence of recurrence: 26% vs. 75%, $p = 0.003$).

3.2.2. Survival analysis

Sixteen published studies [28,33,37,38,41,43–45,47–50,52,68,70,71] reported data on somatic *KRAS* variations and their impact on survival outcomes (Table 2(b)). The same trend between papers published before and after 2016 discussed above for recurrence risk can also be seen for survival analysis. Thus, no statistically significant association between *KRAS* mutations and survival was found in studies published before 2016 (7/16, 43.8%), with the exception of the work by Luna-Pérez et al. [68] which reported a survival benefit for patients with *KRAS* mutations compared to wild-type patients. However, this outlier result could be influenced by the small sample size ($n = 37$), the inclusion of patients with clinical stage IV in the wild-type group, and the use of postoperative surgical specimens to determine *KRAS* mutational status. Of the nine studies conducted after 2016 (9/16, 56.3%), 6 consistently found a significant association between *KRAS* mutations and poor survival; the three remaining studies did not find any association.

The literature search also revealed two meta-analyses that reflect the trend described above. The first meta-analysis by Clancy et al.. (696 patients, 8 studies) [69], published in 2013, confirmed no association between *KRAS* mutations and survival endpoints. On the contrary, the second meta-analysis by Peng et al. (2021) (3674 patients, 16 studies) [51], published in 2021, reported that patients with *KRAS* gene mutations had lower survival compared to wild-type patients (OR:1.33, 95%CI: 1.13–1.56).

The importance of performing subgroup analyses, in which patients are stratified according to molecular characteristics (e.g. specific *KRAS* hotspot mutations), is also evident in survival outcomes. For example, the work of Peng et al.. (2017) [33] reported poorer survival in patients with mutations in *KRAS* gene compared to wild-type patients (wild-type vs. mutated, 3-year survival: 95.4% vs. 88.0%, $p = 0.020$), but a more detailed analysis showed that patients with the *KRAS* subtype G12D (GGT > GAT) (7/70; 10.0%) represented a subgroup with even worse survival than all others (wild-type vs. G12mutated: 95.1% vs. 71.4%, $p = 0.028$). The suggestion to perform a subgroup analysis to better define the role of these somatic variants also came from another work that looked at the links between different signaling pathways. Specifically, Krajnovic et al. [47] found that simultaneous VEGF expression and *KRAS* mutations highlighted a subgroup of patients with poorer survival ($p = 0.001$). In addition, patients who had the specific *KRAS* mutation G12A (GGT > GCT) in codon 12 and high VEGF expression survived throughout the follow-up period. These data emphasize the importance of investigating the predictive effect of specific mutations compared to the assessment of the entire mutational spectrum of the gene. Therefore, in some cases since 2020, the investigation has been conducted using a targeted next-generation sequencing (NGS) approach to clearly determine the exact mutations in the genes of interest. In addition, in more recent works, researchers have not limited the study to the most frequently mutated codons (12–13) but

have also looked for mutations at other codons (e.g. codons 115 and 146) [28,49].

3.3. NRAS

As already mentioned, *NRAS* mutations are not that common, which is why they were often analyzed together with *KRAS* mutations. In most cases, it was not possible to establish a statistical correlation with the endpoint due to the very low frequency of *NRAS* mutations. For example, in the study by Orlandi et al. [35] it was found that among the patients with disease progression (9/39; 23.1%), eight had a *KRAS* mutation, while none carried an *NRAS* mutation, which was present in only one patient in the entire study population (1/39; 2.56%), so a formal analysis was not possible.

Two studies [49,50], although showing a very low *NRAS* mutation frequency (3.51% and 9%), attempted to statistically evaluate the impact of *NRAS* gene mutations on recurrence risk without finding significant differences between wild-type and mutated patients in terms of RFS. The same studies also examined the role of *KRAS* variants on survival, but again no association was found (Tables 1(c) and 2(c)).

3.4. BRAF

BRAF is a gene located on chromosome 7 that encodes a serine/threonine kinase that is part of the MAPK signaling pathway and is involved in the regulation of cell survival, differentiation and growth [24,79]. Like the other members of the RAS family, it is involved in the phosphorylation cascade of MEK and ERK proteins, leading to the activation of numerous nuclear and cytosolic targets. A single mutation in one component of the signaling pathway leads to complete deregulation [80].

Four studies included in this literature review report on the impact of *BRAF* mutations on recurrence risk and survival [38,43,50,53] (Tables 1(d) and 2(d)). These investigations (sample size from 64 to 210 patients) are characterized by a high degree of heterogeneity, which makes a comparison between them very difficult. For example, in three of these studies, *BRAF* mutational status was determined in post-treatment samples in addition to pre-treatment samples, leading to a bias in the interpretation of the results [38,50,53]. In addition, one of the three studies also included patients with stage IV disease, which represents an additional confounding factor for the comparison of the data obtained [38]. Another difference concerned the genotyping method and the panel of mutations analyzed. In particular, the study by Davies et al. [38] focused only on the T1799A transversion in exon 15, which results in a substitution of valine for glutamic acid in codon 600 (V600E). This is the best known *BRAF* mutation, which leads to constitutive activation of the protein kinase domain and accounts for ~90% of all *BRAF* mutations [81]. In this work, genotyping was performed by pyrosequencing and only 3/64 (4.69%) LARC patients with V600E mutation were identified, confirming the low frequency of this alteration in rectal cancer as observed in CRC [82]. The more recent study by Derbel et al. extended the analysis to the entire exon 15 using the Sanger sequencing method and found 2 out of 98 (2.04%) patients carrying *BRAF* mutations [43]; only one of the two mutated patients had the V600E variant, confirming its relatively low frequency. The two other studies included in this review also found the very rare *BRAF* missense mutation D594G (A1781G) in addition to the V600E variant through NGS-based screening [50,53]. This functionally relevant variant is defined as a kinase-dead mutation because it causes the replacement of an aspartate by an aliphatic glycine residue in a highly conserved motif that is responsible for the catalytic activity of BRAF. In the study by Jiang at al [53], 4/74 (5.41%) patients were *BRAF* mutated, 3 (75.0%) with the V600E and 1 (25.0%) with the D594G variant; genotype data were obtained by target NGS and then confirmed by Sanger sequencing. Similarly, in the study by Sclafani et al. [50], 7 of 202 included patients were *BRAF*-mutated (3.47%), with 5/7 (71.4%) patients carrying the V600E variant and 2/7 (28.6%) carrying the D594G; mutational analysis was performed by an NGS approach coupled with microarray method.

Overall, as discussed in the following two paragraphs, it was difficult to find a significant correlation between *BRAF* status and patient outcome due to the low frequency of *BRAF* mutations. This limitation combined with the small number of published articles makes it difficult to define the role of *BRAF* mutation status in LARCs, and further studies with a large population are needed to better determine its prognostic significance.

3.4.1. Recurrence risk

Due to the low frequency of *BRAF* mutations, the four published studies (Table 1(d)) should only be regarded as hypothesis-generating value and the results obtained of a descriptive nature [38,43,50,53]. In particular, in the studies by Davies et al. [38] none of the three patients with *BRAF* V600E mutation experienced recurrence (wild-type vs. mutated: local recurrence, 10% vs. 0%; distant recurrence, 20% vs. 0%), whereas in Derbel et al. [43] of the two patients carrying any *BRAF* mutation, one patient developed local recurrence and the other distant recurrence (wild type vs. mutated: local recurrence, 87.5% vs. 12.5%; distant recurrence: 94.1% vs. 5.9%). The most recent study by Jiang et al. [53] found four *BRAF*-mutated patients in a cohort of 74 LARC samples; *BRAF* mutation was significantly correlated with shorter PFS ($p = 0.045$). In contrast, the study by Sclafani et al. [50] found no differences in PFS between patients with *BRAF*-mutated and *BRAF* wild-type tumors (wild-type vs. mutated, 5-year PFS: 67% vs. 43%, $p = 0.36$).

3.4.2. Survival analysis

As with the recurrence risk, only preliminary data were provided for survival by the four eligible papers [38,43,50,53].

Three of the four works failed to demonstrate a significant difference in overall survival depending on *BRAF* mutational status (Table 2(d)) [38,43,50]. Although this finding is not statistically significant, it should be noted that in the study by Davies et al. [38] the median OS in the wild-type group was 4.1 years, while in the *BRAF*-mutated group the median OS was not reached, as none of the three mutated patients died during the follow-up period. Moreover, in the recent study

by Sclafani et al. [50], overall survival did not differ by mutational status, but a subgroup analysis that only included patients who were not treated with cetuximab showed that patients with *BRAF*-mutated tumors had poorer 5-year survival (wild-type vs. mutated: 73% vs. 20%, $p = 0.03$). Only one study (Jiang et al.) generated positive results indicating that *BRAF*-mutated LARC patients had a shorter OS than wild-type ($p < 0.001$) [53].

3.5. PIK3CA

PIK3CA is another gene that is being investigated for mutations in colon and rectal cancer, particularly because of its close association with the RAS/RAF/MAPK signaling pathway mentioned above. It encodes the alpha-catalytic subunit of the enzyme phosphatidylinositol 3-kinase (PI3K) of the AKT/mTOR pathway, which controls tumor proliferation, differentiation and survival [83]. Approximately 20% of rectal cancers have an activating mutation in the *PIK3CA* oncogene, with hot spots mainly located in exons 9 and 20 [84,85].

The literature search revealed five studies [33,43,50,54,55], all focusing on mutations in exon 9 and 20 detected by different genotyping methods such as direct sequencing of the gene, targeted assay (Sequenom Massarray System Oncocarta Array), NGS or polymerase chain reaction (PCR) assay (Tables 1(e) and 2(e)). The studies by He et al. [54] and Byun et al. [55] were conducted exclusively on post-treatment samples collected at the time of surgery. The first analysis included 240 LARC patients, of which 19 (7.92%) had a mutation of the *PIK3CA* gene, while the second analyzed 109 patients and found three mutations (2.75%) [54,55]. In contrast, the studies by Derbel et al. [43] and Peng et al. [33] obtained mutational data from formalin-fixed paraffin-embedded (FFPE) biopsy samples prior to treatment. These two studies were characterized by a smaller sample size with mutation frequencies of 23.5% (23/98) and 14.3% (10/70), respectively [33,43]. The work by Sclafani et al. [50] was the only one that analyzed both pre- and post-treatment samples and found a *PIK3CA* mutation frequency of 8.82% (18/204).

3.5.1. Recurrence risk

All five eligible studies investigated the effects of *PIK3CA* mutations on recurrence risk [33,43,50,54,55] (Table 1(e)). The first study by He et al. [54] showed that patients with *PIK3CA*-mutated LARC tumors had a threefold increased risk of local recurrence compared to wild-type tumors (wild-type vs. mutated, 5-year risk: 9.4% vs. 27.8%, $p = 0.008$). This study also found a shorter median interval between surgery and local relapse in mutated patients compared to wild-type patients (7.9 vs. 19.6 months) [54]. In addition, a subgroup analysis that excluded patients who received adjuvant therapy confirmed the higher risk of local recurrence for patients with *PIK3CA*-mutated tumors (wild-type vs. mutated, 5-year risk: 6.4% vs. 27.6%, $p = 0.004$) [54]. In the evaluation of distant recurrence, a correlation between the PIK3CA status and the clinical outcome was demonstrated in the work by He et al. [54] (wild-type vs. mutated, 5-year risk: 26.4% vs. 37.1%, $p = 0.413$). Overall recurrence risk resulted also not associated with *PIK3CA* mutational status ($p = 0.107$). He et al. [54] also conducted an analysis that focused specifically on the E545K (G1633A) mutation in exon 9. This mutation is one of the most common *PIK3CA* alterations affecting the helical PIK domain and causing gain of function. This subanalysis revealed that the E545K mutation, harbored by nine patients, was significantly correlated with an increased risk of both local (HR = 11.6; $p < 0.001$) and distant recurrence (HR = 3.7; $p = 0.006$), suggesting that this somatic alteration causes higher tumor aggressiveness [54].

The encouraging results of He et al. [54] were confirmed by the recent work of Byun et al. [55] in which patients with a *PIK3CA* mutation had shorter DFS compared to wild-type patients ($p = 0.006$). In these two studies, the mutational analysis was performed on postoperative specimens [54,55]. In contrast, the other studies did not confirm this result. For example, Derbel et al. [43] found no association between the PIK3CA mutation and local or distant recurrence. Specifically, in the wild-type group ($n = 94$), 8 patients developed local recurrence and 16 developed distant recurrence, while in the mutated- group ($n = 4$), none had local recurrence and only one had distant recurrence ($p = 0.539$) [43]. The retrospective analysis by Peng et al. [33] also showed no significant association between 3-year DFS and mutation status of the *PIK3CA* gene (wild-type vs. mutated: 82.8% vs. 68.6%, $p = 0.632$). Similarly, the work of Sclafani et al. [50] found no association between 5-year PFS and the presence of *PIK3CA* mutations (wild-type vs. mutated: 67% vs. 67%, $p = 0.48$).

3.5.2. Survival analysis

With regard to the survival endpoint, four of the five published studies [33,43,50,54] found no correlation with *PIK3CA* status. Only the most recent study by Byun et al. [55] observed a significantly shorter cancer-specific survival in patients with *PIK3CA* mutation than in wild type (Table 2(e)).

3.6. TP53

The *TP53* gene produces the product p53, a transcription factor that plays a crucial role in tumor suppression by triggering cell cycle arrest, senescence, apoptosis or DNA repair when cells are exposed to various types of cellular stress, such as DNA damage caused by radiotherapy [86,87]. *P53* is the most frequently mutated gene in cancer, with the highest mutation prevalence (43.0%) found in colon cancer [86]. *P53* mutations also occur more frequently in rectal cancer. A paper by Chang et al. reported that 93.1% of patients with rectal cancer had a *TP53* mutation [85]. The role of mutations is better defined in CRC, while it remains to be clarified in rectal cancers, especially LARCs [31].

Although *TP53* is one of the most studied genes, only three articles investigated the association between *TP53* mutations and prognosis and met the eligibility criteria for this review (Tables 1(f) and 2(f)) [50,56,57]. Two of these papers had a sample size of approximately 60 LARC patients and were not recent as they analyzed tissue from patients enrolled between 1991 and 1998 [56,57]. This time of enrollment may represent a bias related to treatment regimen, as patients enrolled before 1994 received preoperative

radiotherapy and only after 1994 patients received preoperative concurrent chemoradiotherapy [57]. There is also some heterogeneity in the method of analysis. The study by Kandioler et al. [56] was performed only on preoperative FFPE samples and analyzed all exons from 2 to 10 by direct sequencing. In contrast, the work of Saw et al. [57] focused on specific exons (4, 5, 6 and 7) of the *TP53* gene, and when the pre-treatment biopsy was not available, analyses were performed on the post-treatment surgical specimens. In this study, the technique used to assess *TP53* gene mutations was PCR amplification with specific primers followed by single-strand conformation polymorphism analysis [57]. In the more recent work by Sclafani et al. [50], 205 patients enrolled in two different phase II trials (Expert-C and Expert) between 2001 and 2008 were genotyped. The exons from 4 to 11 or from 4 to 9 were examined by capillary electrophoresis in combination with Sanger sequencing as confirmation or by targeted NGS.

In the three studies examined, the mutation frequencies were between 45% and 60% [50,56,57].

3.6.1. Recurrence risk

The three studies consistently found no correlation between *TP53* mutational status and the risk of recurrence [50,56,57]. However, the study by Saw et al. [57] which examined each exon individually, found that in the subgroup of patients receiving concurrent chemoradiotherapy, the presence of mutations in exon 5 correlated with local recurrence (Table 1(f)). In the group with normal exon 5, no patient developed recurrence, while 2 of the 6 patients with mutated exon 5 developed recurrence ($p = 0.03$) [57]. Similarly, a trend was observed between mutations in exon 8 and LR: 6 of 49 patients had local progression in the subgroup with normal exon 8 compared to 2 of 3 in the group with mutations in exon 8 ($p = 0.058$) [57].

3.6.2. Survival analysis

With regard to survival, Saw et al. [57]and Sclafani et al. [50] failed to demonstrate an association between *TP53* mutation status and survival, while Kandioler et al. [56] found an increased survival rate in patients with wild-type *TP53* ($p = 0.049$) (Table 1(f)). However, the results obtained by Kandioler et al. [56] using direct sequencing were not confirmed by immunohistochemical analyses.

3.7. MSI status

MSI is a consequence of a defective DNA mismatch repair (MMR) system. This evolutionarily highly conserved system consists of many enzymes that work together to repair DNA damage and ensure the maintenance of genomic integrity and stability [88]. This system of proteins is encoded by four major genes: mutL homologue 1 (*MLH1*), postmeiotic segregation increased 2 (*PMS2*), mutS homologue 2 (*MSH2*), and mutS 6 (*MSH6*) [89]. Germline mutations in MMR genes are responsible for Lynch syndrome, while somatic mutations and epigenetic alterations lead to sporadic CRC [90].

Many studies in the literature have reported the lack of efficacy of fluoropyrimidine-based therapy in patients with deficient mismatch repair (dMMR) colon cancer, especially in stage II [91,92]. Recently, a study by Cohen et al. [93] reported that the addition of oxaliplatin to fluoropyrimidine improved OS and DFS in patients with stage III MSI colon cancer. However, the prognostic value of MSI in LARCs treated with fluoropyrimidine-based chemotherapy in combination with radiotherapy remains uncertain. Certainly, determining MSI status in LARC will be critical, as emerging evidence suggests that durable responses can be achieved when LARC patients with dMMR are treated with immune checkpoint inhibitors against programmed cell death 1 (PD-1) receptor [94].

Ten studies were included in the analysis that attempted to define the relationship between MSI and recurrence risk or survival in LARC [49,58–60,62,63,65,72,73,95] (Tables 1(g) and 2(g)). Compared to the other genes included in this literature review, all of the papers that assessed the prognostic role of the MMR system were recent (from 2013 and later), suggesting that this topic has only recently begun to be studied in LARC. The studies were conducted in different countries and many included large numbers of patients (from 296 to 549), with the exception of El Otmani et al. [49] and Bando et al. [64,95] which included fewer than 100 patients. In addition to the 10 studies, a meta-analysis was recently published in 2022 that includes 22 papers, 5 of which are part of our review, with a total of 16,526 patients analyzed [64]. The tumor stage of the study population at diagnosis was mostly homogeneous I- II-III; patients with stage IV disease at diagnosis were only included in the study by Meillan et al. [59]. When looking at the studies that provided information on preoperative treatment, neoadjuvant chemoradiotherapy with fluoropyrimidines or capecitabine emerged as the most common regimen. The only exception was the work by Du et al. [58], in which the included patients only received radiotherapy in order to exclude a possible influence of chemotherapy on the response to treatment depending on the MSI status. Some of the studies considered also included fluoropyrimidines based adjuvant chemotherapy in combination with oxaliplatin and IRI.

In terms of methodology, there is no consistency in the type of samples used, as both pre- and post-treatment tissues were analyzed to determine MSI status, leading to bias in the interpretation of results. Both immunohistochemical staining (IHC) and PCR assays were used to assess MSI status, and some studies opted to use both methods to obtain better molecular characterization of the sample. Loss of MMR protein expression in the IHC analysis or instability of mononucleotide markers (e.g. BAT-25, BAT-26, NR-21, NR-24, MONO-27) in the PCR assay are considered indicative of microsatellite instability. In general, proficient mismatch repair (pMMR) patients without loss of MMR protein expression or marker instability and patients characterized by the instability of a marker, defined as low microsatellite instable (MSI-L), are classified in the microsatellite stable (MSS) category. In contrast, the MSI category includes dMMR and/or highly microsatellite unstable (MSI-H) patients with loss of MMR protein expression and/or instability in two or more markers. The work of Hasan et al. [73] did not follow this classification, as the MSI group included patients with unspecified microsatellite status in addition to MSI-H and MSI-L. As can be seen from these considerations, there is certainly some inhomogeneity in the classification of MSI status. The

percentage of microsatellite unstable LARC cancers in the papers included in the review, including the meta-analysis, ranged from 3.8% to 19.3%, with 6 (54.5%) papers reporting a percentage of around 8%. This also corresponds to the frequency reported in the literature for rectal adenocarcinomas, which is around 5% to 10% [94].

3.7.1. Recurrence risk

Six of the ten (60.0%) studies found examined DFS rate as endpoint [58–60,62,63,65]; one meta-analysis was also published [64](Table 1(g)). Three papers and the meta-analysis by Swets et al. reported no association between MSI status and DFS [58–60,64]. On the other hand, three recent analyses showed positive results with a trend toward longer DFS in patients with dMMR [62,63,65].

In some works [58,63], subgroup analyses were performed according to clinicopathologic characteristics (i.e. ypTNM stage). In the work by Wu et al. [63] the association between better DFS and dMMR was observed in patients with ypStage II/III (HR = 0.38; $p = 0.020$), but not in those with ypT0N0/Stage I. However, these data were not confirmed by Du et al. [58] where, in contrast, the authors found that dMMR predicted better DFS only in the subgroup of patients without lymph node metastases (ypN0) (pMMR 79.8% vs. dMMR 100%, $p = 0.040$).

No significant data have been published on other endpoints assessing recurrence risk (i.e. local and metastatic recurrence, overall recurrence and RFS), with the exception of preliminary data from Meillan et al. [59] which showed a statistical association between dMMR and lower recurrence risk in a univariate analysis (pMMR 31.1% vs. dMMR 8.7%). This is also confirmed by the estimation of the 5-year RFS with Kaplan-Meier curves ($p = 0.0463$) [59].

The study by Bando et al. should be discussed separately because it contained a treatment bias, as the patients received the immune checkpoint inhibitor nivolumab, whose efficacy was known to be related to MSI status [95]. This study investigated the impact of MSI status on recurrence in a small cohort of 44 patients and it found that patients with dMMR LARC cancer had a lower risk of relapse. Specifically, 6 patients with pMMR LARC cancer developed recurrence (2 locally and 4 distantly), whereas no patients with dMMR had recurrence [95].

It should be noted that although no overall significant data were found in the Lee et al. [60] paper, an attempt was made to investigate a possible interaction between MSI status and the immune system. It was found that patients with dMMR before CRT had an increased neutrophil-to-lymphocyte ratio and an increased platelet-to-lymphocyte ratio, and the combination of dMMR with high inflammatory markers predicted worse DFS. This preliminary observation suggests a relationship between MSI status and infiltration of the tumor environment by immune cells [60].

3.7.2. Survival analysis

Seven out of ten (70.0%) eligible papers [49,59,60,62,72,73,95] and one meta-analysis [64] investigated the role of MSI status on survival outcomes without finding a significant association (Table 1(g)). Only the REACCT collaborative study indicated an improved OS for patients with a dMMR tumor (MSI vs. MSS, median overall survival: 58 vs. 32 months) [62]. In the study by Bando et al. on patients who also received the immune checkpoint inhibitor, it was reported that one death occurred in the pMMR group while none was recorded in the MSI group, but the small population and short study duration did not allow a formal statistical analysis [95].

3.8. SMAD4

SMAD4 is a gene that encodes a protein involved in the TGFβ signaling pathway and acts as a tumor suppressor. This protein is part of a complex with the other SMAD proteins that migrates from the cytoplasm to the nucleus, where it mediates the transcription of target genes that are critical for cell cycle progression and survival [96]. It was one of the most frequently mutated genes in CRCs (approximately 5–24.2%) [97], and a recent study reported a prevalence of 20.7% in rectal carcinomas [85].

3.8.1. Recurrence risk and survival analysis

For *SMAD4*, only one study was included in this literature review [53] (Tables 1(h) and 2(h)). In this work, targeted mutations in exons 9–12 were analyzed by NGS method in a cohort of 74 LARC patients treated with multiple neoadjuvant regimens. This study identified 8 (10.8%) patients with a SMAD4 gene mutation and showed that the group with somatic SMAD4 variations had a shortened PFS ($p = 0.008$) [53]. In the same study, no significant association was found between SMAD4 mutations and survival [53].

3.9. Multiple mutations

Given the interplay of multiple biological pathways in determining tumor behavior and potential response to nCRT, simultaneous analysis of multiple genes may be a more effective strategy than analysis of a single gene. Some papers that met the eligibility criteria for this literature review attempted to investigate the prognostic impact of simultaneous mutations in multiple genes [50,54,66,67] (Table 1(i)).

For example, the previously cited paper by He et al. [54] examined the combined mutation of *PIK3CA* and *KRAS*. Of the 19 subjects who had a *PIK3CA* mutation, 10 subjects also had a *KRAS* mutation, which corresponded to 4.2% of the total cohort of 240 patients. The two genes encode proteins that are closely linked, and the presence of simultaneous mutations can alter all downstream signaling pathways and impair cell proliferation and survival. Of note, concurrent mutations occurred in 8% of female versus 2% of male patients and their presence significantly affected the rate of local recurrence, with a 5-year risk of 33.3% in double mutated patients versus 10.0% in single or non-mutated patients ($p = 0.012$) [54]. These percentages were similar to those reported for local recurrence when only the PIKCA mutations were analyzed, suggesting that mutations on the *PIK3CA* gene appear to be the major contributor. In contrast, there was no association between

distant recurrence and concurrent *PIK3CA* and *KRAS* mutations [54].

Oshiro et al. [66] investigated the RAS/RAF mutational status in 57 LARC patients treated with fluoropyrimidines in combination with oxaliplatin with or without bevacizumab. In the study, patients were divided into RAS/RAF wild-type (23/57, 40.3%) and RAS/RAF mutated (34/57, 59.6%); no concurrent mutations were present, confirming that RAS/BRAF mutations are mutually exclusive [66]. The analysis showed that the group with mutated tumors had a worse 3-year RFS (59%) than the wild-type group (95%) [66]. It is noteworthy that no patients in this study received preoperative radiotherapy [66].

The study by Kamran et al. [67] characterized the whole exome mutations of KRAS and TP53 genes using NGS in a cohort of 17 LARC patients treated with fluoropyrimidine-based nCRT. This work confirmed that *TP53* (13/17, 76.5%) and *KRAS* (10/17, 58.8%) were the most frequent somatic alterations also in LARC samples [67]. Moreover, the analysis of FFPE samples matched before and after treatment showed a dynamic evolution of the mutational profile that should be taken into account in the search for pharmacogenomic markers. In particular, 7 out of 17 patients (41.2%) were found to have both *KRAS* and *TP53* mutations [67]. Of these 7 patients, six had a tumor with a mutated *KRAS/TP53* genotype in both pre- and post-treatment samples, while one patient had the mutation only in the post-treatment sample, suggesting that he acquired the mutation during therapy. The analysis showed that patients with tumors that had a mutated *KRAS* and *TP53* genotype in the pre- and/or post-treatment samples had a lower 5-year PFS than the other patients (wild-type vs. *KRAS/TP53*-mutated: 90% vs. 38%, $p = 0.04$) [67].

The study by Sclafani et al. [50] investigated the effects of the simultaneous presence of *TP53* and *KRAS/NRAS*. In contrast to the previous work, this study did not examine the entire exome, but only certain exons (4 to 11 for TP53 and 2 to 4 for *KRAS* and *NRAS*) [50]. The *TP53/KRAS/NRAS* genotype was observed in 63 of 199 (31.7%) evaluable tumors. Patients with LARC tumors harboring these mutations had a worse 5-year PFS than patients with wild-type tumors (wild-type vs. mutated; 72% vs. 54%, $p = 0.02$) [50].

4. Conclusions

Considering the various therapeutic options available for the treatment of LARC patients in recent years, choosing the most appropriate therapeutic approach based on patient risk stratification has become a crucial step in the treatment of LARC patients. Therefore, in the era of precision medicine, there is an urgent need to identify new molecular markers that can be combined with clinicopathological and possibly radiological features to achieve better prognostic stratification of patients. The present review of the literature suggests that the presence of a *KRAS* mutation may be a risk factor for poor prognosis in LARC patients treated with preoperative radiation-based regimen. These findings encourage the inclusion of *KRAS* mutations in the decision algorithm for selecting the most appropriate treatment strategy for LARC patients to avoid surgery-related complications or unnecessary treatments and improve patients' quality of life, which is also beneficial for the healthcare system.

5. Expert opinion

With the recent introduction of immunotherapy as an option for the preoperative treatment of LARC patients, testing for MSI status is currently required prior to drug administration. However, in contrast to colorectal cancer, where some drugs are selected on a molecular basis, in rectal cancer – with the recent exception of MSI status – there are currently no validated biomarkers that influence the choice of the most appropriate treatment, and fluoropyrimidine-based preoperative therapy is administered regardless of molecular background. Over the last decade, several pharmacogenomic studies have investigated the role of somatic mutations in key oncogenes such as *RAS, TP53, BRAF, PIK3CA and SMAD4* as well as MSI status in predicting the risk of local recurrence/distant metastasis and survival probability in LARC patients to define potential biomarkers of clinical utility. Overall, these studies are very heterogeneous, making it difficult to compare the literature data. The inhomogeneity of the published results is due in particular to different study designs (e.g. retrospective/prospective analyzes) and treatment regimens (e.g. radiotherapy only or standard chemoradiotherapy or intensified chemoradiotherapy, type of fluoropyrimidines administered and possible simultaneous administration of other therapeutic agents, radiotherapy dose, interval until surgery, type of surgery, possible administration of adjuvant therapy) as well as the heterogeneity of the clinical and demographic characteristics of the patients (e.g. gender, age, ethnicity), differences in the definition of the clinical endpoints and in the measurement of the clinical parameters, and variations in the analytical methods used to investigate the molecular marker candidates. Another important aspect is that most of the published studies have a very small sample size, which has a negative impact on statistical power and thus the generation of robust data. In addition, although most studies include stage II-III patients, there are also studies that include stage I and IV patients, which could have a significant impact on the assessment of prognostic outcomes such as relapse risk and survival probability. It should also be considered that in some published analyzes molecular data were obtained not only from pre-treatment biopsies but also from post-treatment biological samples, and this aspect could introduce a bias related to the effects of chemoradiotherapy on tumor biology (e.g. induced change in the mutational pattern, selection of resistant cell clones or complete disappearance of tumor cells in patients with a pCR). However, despite these limitations, the published studies may provide some interesting insights into potential pharmacogenomic markers that can be used in the therapeutic management of LARC patients.

The present literature search has shown that somatic mutations in the *KRAS* gene are a reliable marker for a poor prognosis and could play a role in clinical practice. In particular, most of the recently published studies have consistently reported an association between mutated *KRAS* and an increased risk of local and/or distant recurrence and shorter survival in LARC patients treated

with radiation-based preoperative treatment. In 2011–2013, the inclusion of cetuximab in preoperative treatment regimens in LARC patients was investigated. In this context, cetuximab was administered to all patients, regardless of KRAS mutation status. The association between mutated *KRAS* and poor prognosis was most evident in studies where cetuximab was not included in the preoperative treatment regimen, which is consistent with previously published data [31], and confirms that KRAS mutation has no impact on response to the specific anti-EGFR drug. The negative impact of *KRAS* mutations on the prognosis of LARC patients observed in the present review is consistent with the results of a recent meta-analysis showing that patients with mutated *KRAS* have an increased risk of not responding to preoperative treatment (no pCR) [31]. Thus, given the predictive and prognostic value emerged for *KRAS* mutations in LARC, biomolecular assessment of *KRAS* status should be integrated into the currently used clinical algorithm for selecting the best therapeutic strategy. In addition, the assessment of *KRAS* status should also be proposed by international clinical guidelines for treatment selection in non-metastatic rectal disease as is already the case for metastatic CRC. For example, in the presence of an unfavorable prognostic factor, including *KRAS* mutations, the decision for a 'watch-and-wait' strategy should be made with caution due to the high risk of disease recurrence, while intensified preoperative treatment might be a better strategy. It would be interesting to conduct further studies to investigate whether certain *KRAS* codon mutations have a particular impact on treatment outcome in LARC patients, as suggested by the recently published preliminary data [35,49].

It is still unclear whether or not the effects of *KRAS* status on the outcome of LARC patients depend on treatment, and further analyzes should clarify this pending issue [25]. Moreover, combining *KRAS* mutational status with additional molecular features (e.g. somatic mutations in *TP53* or *BRAF* genes, vascular endothelial growth factor expression) in a polygenic risk score that takes into account the interplay of multiple pathways should further improve the prognostic risk stratification of LARC patients [47,66,67].

Despite its potential interplay with radiotherapy and chemotherapeutic agents (e.g. 5-fluorouracil) in modulating the tumor microenvironment and immune response, MSI status does not appear to be a significant predictor of patient prognosis, both in terms of recurrence risk and survival probability, according to the present literature review. Only a trend for a better DFS in patients with dMMR tumors could be inferred from the data, but further analyses with a more homogeneous study design and study population are needed. It should be noted, however, that MSI status has recently gained attention in LARC treatment with the introduction of immune checkpoint inhibitors as an option for the neoadjuvant treatment of LARC patients. Some preliminary trials have reported a benefit of immunotherapy, particularly for patients with dMMR LARC, with an improvement in response to neoadjuvant therapy and the possibility of avoiding chemoradiotherapy and/or radical surgery [61,94,98]. There is considerable research interest in determining the potential of immune checkpoint inhibitors in neoadjuvant setting both in colon and rectal cancer. The results of recent trials (e.g. NICHE, NICHE-2) suggest that neoadjuvant immunotherapy has the potential to become a new standard of care in dMMR and possibly a subset of early-stage pMMR colon cancers, which requires further validation in larger studies currently underway [99–101]. Similarly [94], several ongoing clinical trials, including the VOLTAGE-2, evaluating the efficacy of immune checkpoint inhibitors in patients with dMMR LARC have reported outstanding responses [102].

As for the other genes investigated (i.e. *TP53, BRAF, PIK3CA* and *SMAD4*), the small number of published studies together with the heterogeneity of the data obtained does not allow a definitive conclusion on a possible role of mutations in these genes as clinically useful pharmacogenomic markers.

It should be reported that, in addition to the genes considered in the present review, other genes (e.g. FBXW7 and SOX9) are also frequently mutated in rectal cancer [85,103–105] but, currently no studies are available investigating their potential role as predictive markers for response to neoadjuvant radiation-based therapy in patients with LARC. Moreover, some biomarkers that are routinely tested in the clinical care of CRC, such as HER-2 gene expression, currently play a less defined role in rectal cancer. For example, there is limited data on the role of HER2 expression in predicting the prognosis of LARC patients treated with neoadjuvant radiation-based therapy. The prevalence of HER2-positive rectal tumors is low [28,106] and HER2 expression does not appear to be significantly related to the clinicopathologic and molecular characteristics of rectal tumors [106]. Only one preliminary study has reported an association between HER2 expression and tumor recurrence [49], and further evidence are required before HER2 can be considered a potential marker of clinical benefit in LARC patients.

Another point to consider is the timing of molecular testing. Indeed, a dynamic evolution of the mutational pattern of LARC tumors during neoadjuvant therapy has been described by evaluating samples before and after treatment [67,107,108]. In particular, several differences in the genomic landscape of rectal cancer were described between paired samples pre- and post-chemoradiotherapy, suggesting that the treatment could alter the genomic features of tumors both at the levels of somatic mutations and copy number variations [67,107,108].

This aspect, which underlines the importance of a homogeneous and standardized timing of sampling, should be considered when discovering pharmacogenomic markers and planning the use of the same markers in clinical practice to optimize LARC treatment. As stated before, the molecular data discussed in the present review were obtained from both pre- and post-treatment biopsies; this represents a limitation and emphasizes the need to control for this confounding factor in future studies.

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Declaration of interest

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