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A systematic review

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BACKGROUND: *Colorectal cancer (CRC) is one of the leading cause of cancer deaths worldwide. The aetiology of CRC is complex and involves interaction on environmental and genetic factors. The two most important pathways are the EGFR (Epidermal Grow Factor Receptor) signaling pathway, with the involvement of KRAS and BRAF, and the DNA mismatch repair (MMR). Generally, KRAS and BRAF mutations are mutually exclusive. They are both able to cause RAS/RAF/MAPK signaling pathway upregulation and are necessary for CRC development. BRAF mutations confers a poor prognosis in Western CRC patients, particularly in metastatic CRC (mCRC) and its mutations occur in approximately 4-20% CRC, with the vast majority being the V600E hotspot mutation. KRAS mutations are observed in 30-40% CRC patients and act as predictive markers of resistance to epidermal growth factor receptor (EGFR)-targeted antibodies in metastatic CRC. Initial patient management is defined by TNM stage at diagnosis but in patient with stage II and III CRC, TNM staging alone does not predict outcome in CRC patients who may be eligible for adjuvant chemotherapy. Furthermore, for stage II and III, non-metastatic CRC patients, the prognostic role of BRAF and KRAS mutations is still controversial, particularly comparing microsatellite-unstable (MSI) and - stable tumors (MSS). The aim of this study was to clarify the impact of KRAS/BRAF mutations on prognosis in patients with stage I-III CRC.*
MATERIALS AND METHODS: *A systematic review of literature was undertaken to evaluate the prognostic value of KRAS and BRAF mutations in stage I-III colorectal cancer. Four major databases (PUBMED, EMBASE, WEB OF SCIENCE and COCHRANE LIBRARY) were searched.*
RESULTS: *Ninety-two studies were identified. After screening of titles, abstract and inclusion criteria sixteen articles were included. Of the selected articles, five were prospective, ten were retrospectives studies, and one was a combined retrospective/prospective study.*
CONCLUSION: *In our opinion, a combination of molecular markers, tumor location with the other clinical-pathological variables and microsatellite status is essential to have a correct prognosis. Nevertheless, this combination could be useful as a predictive factor in stage I-III CRC.*

KEY WORDS: BRAF, Colorectal Cancer, KRAS, Stage I-III CRC, Translational research

Introduction

Colorectal Cancer (CRC) is the third most common cancer and the fourth most common cause of cancer death in the world [1].

It is a complex biological process driven by genetic [2] and epigenetic alterations [3-5] that regulate proliferation, apoptosis and angiogenesis.
According to this assumption, molecular testing is currently recommended before any treatment in Western countries [6,7].
The two most important pathways are the EGFR (Epidermal Grow Factor Receptor) signaling pathway, with the involvement of KRAS and BRAF, and the DNA mismatch repair (MMR) system.
Deficient MMR (dMMR) can result from a germline mutation in a MMR gene (MLH1, MSH2, MSH6,

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PMS2) or can be sporadic due to epigenetic inactivation of MLH1.
KRAS and BRAF mutations are mutually exclusive. They are both able to cause RAS/RAF/MAPK signaling pathway upregulation and are necessary for CRC development.
Approximately 30-40% CRC shows a guanosine triphosphate/guanosine diphosphate binding protein mutations, a protein encoded by KRAS, one of the first genes found to be mutated in human cancer [8,9].
BRAF is a member of the Raf kinase family that is a regulator of the MAP kinase/ERK signaling pathway [10,11]. It encodes a serine/threonine protein kinase, a downstream effector of the KRAS protein, and its mutations occur in approximately 4-20% CRC [12,13], with the vast majority being the V600E hotspot mutation. In particular is found in 5% to 10% of metastatic CRC (mCRC) and is mutually exclusive of KRAS codon 12 and 13 mutations [14]. Despite it does not have a clear predictive role useful to guide the correct therapeutic decision, it has a major prognostic role with poor survival reported in BRAF-mutant mCRC [15]. In addition to the traditional adenoma-to-carcinoma sequence [16,17] an alternative pathway is described when KRAS mutations develop as an early event in proficient MMR (pMMR) cancers [17].
Furthermore, via this serrated pathway a sporadic CRC, characterized by $BRAF^{V600E}$ mutations, can also develop. Microsatellite instability (MSI) occurs in 15% of CRC and results from a genetic or epigenetic defect in DNA MMR, occurring sporadically (12%) or due to Lynch syndrome (3%) [18]. Microsatellite-unstable tumors are associated with better prognosis compared with their stable tumor (MSS) counterparts.
Even though the prognostic impact of KRAS in stage II and III colon cancers has been inconsistent [19-23] it has been well recognized that $BRAF^{V600E}$ mutations confers a poor prognosis in Western CRC patients [24-27], particularly in mCRC [24,28-31].
Previous retrospective observational studies [32,33] as well as randomized controlled trials [34,35] have shown that KRAS in stage IV CRC confers resistance to anti-EGFR targeted treatment confirming its predictive role. Recently, also $BRAF^{V600E}$ mutation were associated with tumor resistance to EGFR-targeted antibodies [36,37].
Initial patient management is defined by TNM stage at diagnosis, based on depth of tumor wall invasion, lymph node involvement and distant metastasis. This staging system is simple, useful and associated with 5-year overall survival (OS), ranging from 92% in stage I to 11% in stage IV [38].
In patient with stage II and III CRC, TNM staging alone does not predict outcome in CRC patients who may be eligible for adjuvant chemotherapy. Furthermore, it is inconclusive whether TNM stage II colorectal cancer requires adjuvant chemotherapy after radical resection because the factors that place a patient in a high-risk category are subjective descriptive indicators depending on the quality of the initial pathological diagnostic report [39,40]. In stage III colon cancer, post-operative adjuvant chemotherapy is the international standard of care for improved survival [41,42]. Similarly, radiotherapy combined with chemotherapy is recommended as a standard adjuvant therapy for rectal cancer [42,43].
For this reason, new screening programs and several studies are required for early diagnosis of CRC.
The aim of this systematic review is to evaluate if cancer cell molecular markers ($BRAF^{V600E}$ mutations and KRAS mutations) can be useful as prognostic factors, improving traditional clinical-pathological variables in patients with stage CRC.

Materials and Methods

Search Strategy for Identification of Studies

A comprehensive search of four major databases (Pubmed, Embase, Web Of Science And Cochrane Library) was performed on January 2018. The main keywords used for the search were KRAS/BRAF, $BRAF^{V600E}$, MSI, MSS, MMR, molecular markers, stage I-III colorectal cancer, CRC, prognosis or survival. Only articles in the English language were considered for review. Two authors (GG and GS) screened manually and independently citation lists of retrieved articles. All selected studies were checked according to a Newcastle-Ottawa Quality Assessment [44] which was developed previously.

Study Selection

The inclusion criteria were as follows: (1) independently published randomized trial investigating the association between KRAS/BRAF mutations detected by cancer specimen and survival in cancer patients; (2) studies reporting the association between KRAS /BRAF mutation and survival in cancer patients; (3) studies reporting stage I-III colorectal cancer patients curatively resected. Instead the exclusion criteria were: (1) abstracts and reviews; (2) studies without enough information; and (3) repeated or overlapping publications.

Assessment of Methodological Quality of Studies

Two investigators (GG and GS) performed independently data extraction and quality assessment. They collected the detaliled informations of each elegible studies (first author name and institution, year of publication, period of study, number of patients and baseline characteristics, the age of study population, country of study, ethnicity, incidence of KRAS and BRAF mutations, microsatellite instability, methods and type of mutations,

neoadjuvant and adjuvant therapy, duration of follow-up, cancer types, stage disease, survival statistics and HR estimates). When we found overlapped studies, we selected the most recently published study or studies with the largest numbers of subjects to be further analyzed. In addition, the identified articles were examined to identify additional relevant publications.
The nine-star Newcastle–Ottawa Scale (NOS) [44] was performed to assess the quality of each eligible study. With a NOS score equal or greater than seven, a study would be considered to be with high quality.

Data Collection and Statistical Analysis

Efficacy measures report the association between KRAS/BRAF mutations, MMR and survival in cancer patients. Statistical analyses were performed only on the

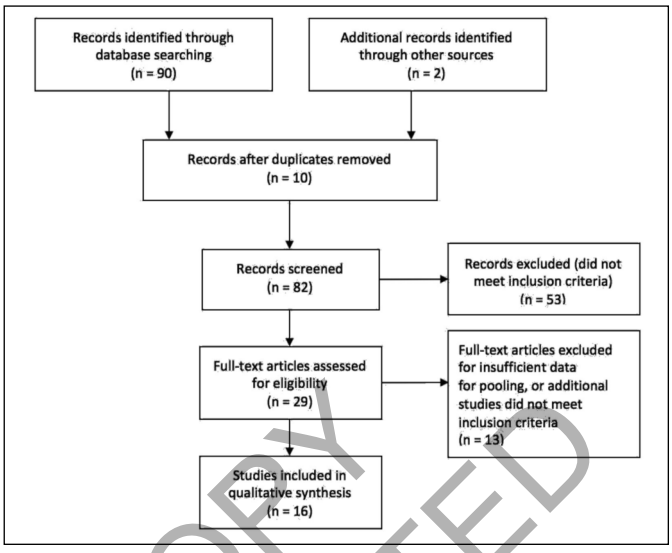

Fig. 1: PRISMA flow diagram.

Table I - *The main characteristics of the included studies*

References	Country	Methods	Prognostic value	Comments	Type of study
Yoon, *et al.* [46]	USA	N/A	Significant for BRAF and KRAS	Only for blacks younger patients	Prospective
Dienstmann, *et al.* [47]	USA	N/A	Significant for BRAF	Compare MSS/BRAF mut with MSI/BRAR wt	Retrospective
Lochhead, *et al.* [48]	USA	N/A	Significant for BRAF	Significant in MSS status	Retrospective
Ogino, *et al.* [49]	USA	PCR and Pyrosequencing spanning	Not Significant for KRAS	Stage III patients	Prospective
Sinicrope, *et al.* [50]	USA	PCR	Significant for BRAF and KRAS	MSS patients Stage III	Retrospective
Sasaki, *et al.* [51]	Japan	PCR for KRAS Melting analysis for BRAF	Significant for KRAS	Predictive role in patients treated with adjuvant chemotherapy	Prospective
Kadowaki, *et al.* [52]	Japan	Electrophoresis for KRAS PCR for BRAF	Significant for BRAF and KRAS	Stage I-III. Significant only in MSS patients.	Prospective
Taieb, *et al.* [53]	France	PCR	Significant for BRAF and KRAS	MSS patients	Retrospective
Etienne-Grimaldi, *et al.* [54]	France	N/A	Not significant for BRAF and KRAS	BRAF-mutated have a better prognosis	Prospective
Blons, *et al.* [55]	France	PCR	Significant for codon 12 KRAS mutation	KRAS significant in distal tumor	Retrospective-Prospective
Chen, *et al.* [56]	China	PCR	Significant for BRAF and KRAS codon 13 mut	Not significant for KRAS codon 12	Retrospective
Li Li, *et al.* [57]	China	Sanger sequencing	Significant for BRAF and KRAS	Stage II MSS status	Retrospective
Shen, *et al.* [58]	China	PCR	Significant for KRAS codon 61 mutation	Significant for DFS in Stage III alone and for OS	Retrospective
Zlobec, *et al.* [59]	Switzerland	PCR	Significant for BRAF	Significant for right-sided disease	Retrospective
Won, *et al.* [60]	Korea	Sanger sequencing for KRAS. PCR for BRAF	Significant for BRAF. Not significant for KRAS	No difference between MSS/MSI-L and MSI-H	Retrospective
de Cuba, *et al.* [61]	Netherlands	N/A	Significant for BRAF and KRAS	MSI patients No significant in Stage II	Retrospective

N/A = Not applicable

Table II - *Studies included*

Author [Ref]	Patients (n)	T Status (n)	N Status (n)	Stage (n)	Grading (n)	Tumor Site (n)	Chemotherapy (n)	Gender (n)	KRASm (n)	BRAFm (n)	MMR status (n)	Age (Median, years)
Yoon et al. [46]	3305	**T1/T2** (496) **T3/T4** (2807) **N/A** (2)	**N1** (1949) **N2** (1356)	N/A	**G1/G2** (2481) **G3** (824)	**LC** (1578) **RC** (1679) **N/A** (48)	N/A	N/A	N/A	N/A	**MSS** (2811) **MSI** (372) **N/A** (122)	N/A
Dienstmann et al. [47]	8904	N/A	N/A	**IIa** (1730) **IIb/IIc** (238) **IIIa** (582) **IIIb** (3677) **IIIc** (1099)	**G1/G2** (5386) **G3** (1168)	**LC** (3263) **RC** (3281)	**N/A** (1080) **5FU+Capecitabine** (852) **FOLFIRI** (732) **FOLFIRI+Cetuximab** (41) **FOLFOX** (1871) **FOLFOX+Cetuximab** (1335)	**M** (3460) **F** (3084) **N/A** (2360)	2563	941	**MSS** (6167) **MSI** (1159)	62.5
Lochhead et al. [48]	1253	N/A	N/A	**I** (298) **II** (351) **III** (323) **IV** (170) **N/A** (111)	**G1/G2** (1123) **G3** (123) **N/A** (7)	**LC** (384) **RC** (583) **R** (273) **N/A** (13)	N/A	**M** (570) **F** (683)	449	182	**MSS** (1060) **MSI** (193)	68.5
Ogino et al. [49]	508	**T1/T2** (59) **T3** (410) **T4** (33)	**N1** (321) **N2** (184) **N/A** (3)	**IIIa** (49) **IIIb** (270) **IIIc** (184) **N/A** (5)	N/A	**LC** (212) **RC** (291) **N/A** (5)	**5FU+Leucovorin** 266 **IFL** 242	**M** (276) **F** (232)	178	N/A	**MSS** (394) **MSI** (66)	59.8
Sinicrope et al. [50]	2720	**T1/T2** (412) **T3** (1973) **T4** (334) **N/A** (1)	**N1** (1591) **N2** (1129)	**III** (2720)	**G1/G2** (2057) **G3** (663)	**LC** (1332) **RC** (1348)	**FOLFOX ± Cetuximab**	**M** (1438) **F** (1282)	945	189	**MSS** (2465) **MSI** (255)	58.0
Sasaki et al. [51]	304	**T1-T3** (199) **T4** (105)	**N1** (247) **N2** (44) **N3** (13)	**IIIa** (246) **IIIb** (58)	**G1/G2** (280) **G3** (13)	**C** (164) **R** (140)	**Tegafur-Uracil** (152) **N/A** (152)	**M** (168) **F** (136)	134	N/A	**MSS** (281) **MSI** (23)	60
Kadowaki S et al. [52]	813	**T1** (83) **T2** (152) **T3** (486) **T4** (91) **N/A** (1)	**N0** (307) **N1** (505) **N/A** (1)	**I** (183) **II** (322) **III** (307) **N/A** (1)	**G1/G2** (760) **G3** (52) **N/A** (1)	**LC** (338) **RC** (232) **R** (242) **N/A** (1)	**5FU+LV+OX** (361)	**M** (474) **F** (338) **N/A** (1)	312	40	**MSS** (67) **MSI** (745)	64.1
Taieb et al. [53]	4411	**T1/T2** (572) **T3** (3153) **T4** (684) **N/A** (2)	**N1** (2647) **N2** (1764)	N/A	**G1/G2** (3398) **G3** (993) **N/A** (20)	**LC** (2320) **RC** (2018) **LC+RC** (56)	**FOLFOX** (2362) **FOLFOX + Cetuximab** (2049)	**M** (2397) **F** (2014)	1522	480	**MSS** (3934) **MSI** (477)	58.4
Etienne-Grimaldi et al. [54]	251	N/A	N/A	**I** (30) **II** (116) **III** (105)	**G1/G2** (148) **G3** (88)	**LC** (122) **RC** (95) **R** (33)	**FUFOL** (47) **FOLFOX** (6) **FOLFIRI** (4) **5FU + Leucovorin** (29)	**M** (150) **F** (101)	N/A	23	**MSS** (205) **MSI** (32)	N/A
Blons et al. [55]	1657	**T1** (46) **T2** (129) **T3** (1143) **T4** (347) **N/A** (2)	**N1** (1051) **N2** (606)	**III**	**G1/G2** (1354) **G3** (283)	**LC** (1043) **RC** (586) **LC+RC** (20)	**FOLFOX ± Cetuximab**	**M** (960) **F** (697)	638	N/A	N/A	60
Chen et al. [56]	214	N/A	N/A	**I** (32) **II** (78) **III** (82) **IV** (19) **N/A** (3)	**G1/G2** (192) **G3** (7) **N/A** (15)	**C** (126) **R** (88)	N/A	**M** (127) **F** (87)	96	9	N/A	68
Li Li et al. [57]	160	N/A	N/A	**IIa** (112) **IIb** (19) **IIc** (29)	**G1/G2** (117) **G3** (53)	**LC** (35) **RC** (43) **R** (82)	N/A	**M** (104) **F** (56)	73	10	**MSS** (139) **MSI** (21)	N/A
Shen et al. [58]	228	**T2** (7) **T3** (207) **T4** (13) **N/A** (1)	**N0** (124) **N1** (58) **N2** (45) **N/A** (1)	**II** (124) **III** (104)	**G1/G2** (191) **G3** (36) **N/A** (1)	**LC** (121) **RC** (107)	N/A	**M** (137) **F** (91)	86	16	N/A	60

(Segue)

(Segue)

Table II - *Studies included*

Author [Ref]	Patients (n)	T Status (n)	N Status (n)	Stage (n)	Grading (n)	Tumor Site (n)	Chemotherapy (n)	Gender (n)	KRASm (n)	BRAFm (n)	MMR status (n)	Age (Median, years)
Zlobec et al. [59]	401	**T1/T2** (83) **T3/T4** (312) **N/A** (6)	**N0** (202) **N1/N2** (186) **N/A** (13)	N/A	**G1/G2** (372) **G3** (23) **N/A** (6)	**LC** (262) **RC** (137) **N/A** (2)	N/A	**M** (186) **F** (215)	118	45	N/A	69.5
Won et al. [60]	1092	**T1** (96) **T2** (177) **T3** (658) **T4** (134) **N/A** (33)	**N0** (569) **N1** (290) **N2** (231) **N/A** (2)	**Tis** (23) **I** (204) **II** (307) **III** (398) **IV** (156) **N/A** (4)	**G1/G2** (1003) **G3** (40) **N/A** (49)	**LC** (468) **RC** (275) **R** (328) **Multiple** (22) **N/A** (2)	N/A	**M** (672) **F** (420)	401	44	**MSS** (77) **MSI** (6)	N/A
de Cuba et al. [61]	143	N/A	N/A	**II** (85) **III** (58)	**G1/G2** (95) **G3** (45) **N/A** (3)	**LC** (27) **RC** (114) **N/A** (2)	N/A	**M** (62) **F** (81)	73	23	**MSI** (143)	N/A

N/A = Not applicable Right Colon = RC; Left Colon = LC; Colon = C; Rectum = R; 5FU = 5-Fluorouracil; IFL = Irinotecan, 5FU, Leucovorin; FUFOL = 5-Fluorouracil and Folinic Acid

extracted data from the selected studies. Basic descriptive statistics (simple counts, percentages and means) were used to summarize the characteristics of the included studies.

Results and Discussion

Firstly, literature searches resulted in 92 studies. Then, we excluded 63 records because of the duplications or no information on KRAS/BRAF mutations and survival in cancer patients through the screening of the titles and abstracts of all studies. The rest of 29 records were screened by full texts. Lastly we included 16 only studies in our systematic review as shown in the preferred reporting items for systematic Review and Meta-Analysis (PRISMA) [45]. The selection process for the eligible studies was shown in Fig. 1. The main characteristics of the eligible studies were summarized in Table I.

The Main Characteristic of the Studies Included

Among 16 included studies, all patients were treated with adjuvant therapy and all studies reported the association of KRAS/BRAF mutations detected on cancer specimen with OS and Progression Free Survival (PFS) in cancer patients. In all studies MSI was assessed. Of these 16 studies (Table I) the majority were from USA (5) [46-50] and the others were from Japan (2)[51,52], France (3)[53-55], China [56-58], Swiss [59], Korea [60] and Netherlands [61]. Regarding study design, five were prospective [46,49,51,52,54,] ten were retrospectives studies [47,48,50,53,56-61] and one was a combined retrospective/prospective study [55]. Baseline data are summarized in table II. Due to the heterogeneity, the meta-analysis of the included studies was not possible.

Patient Characteristics

The data set consisted of 16 studies involving 27460 patients. Table II reports the details of each study.

The most consistent method of reporting disease stage at presentation was using the American Joint Committee on Cancer tumor node metastasis (TNM) staging system [62]. The patients involved in the review had CRC TNM stage I-III disease. In the majority of studies mutation status has been stated using genomic DNA extracted from macrodissected tumor tissue collected prospectively and testing for the $BRAF^{V600E}$ mutation in exon 15 has been perfomed using a multiplex allele-specific, real-time polymerase chain reaction (RT-PCR)-based assay and an automated sequencing technique.

KRAS status has been analyzed in extracted DNA using (RT-PCR)-based assay in codons 12. In one study [57] Sanger sequencing was used to detect the mutations in exons 2 and 3 of *KRAS* and exon 15 of BRAF. In two studies [49,61] PCR and pyrosequencing spanning KRAS codons 12 and 13 was performed. MMR proteins (MLH1, MSH2 and MSH6) expression was analyzed in formalin-fixed, paraffin-embedded tumor sections. Monoclonal antibodies included mouse anti-human MLH1, anti-human MSH2 and anti-human MSH6. Administration of adjuvant chemotherapy was documented in nine studies [47,49,50-55,61].

The aim of this systematic review is to investigate the prognostic value of KRAS/BRAF mutation detected on survival in CRC patients. Eleven studies investigated simultaneously the prognostic role of KRAS/BRAF mutated. In nine studies [47,50,52,53,56,57,59,61] BRAF mutation was associated with a poorer survival in CRC patients and in three studies KRAS [54,56,60] failed to correlate with prognosis. Furthermore, in two studies BRAF [54,60] was irrelevant as a prognostic factor.

The results are that nowadays the clinical management of CRC is still based on clinic-pathological staging.
In particular, Chen, *et al.* [56] included in their retrospective study 214 patients with CRC, demonstrating that patients with $BRAF^{V600E}$ mutations were associated with advanced TNM (P <0.001), more distant metastases (P = 0.025), and worse OS (P < 0.001; multivariate HR = 4.2, P = 0.004). Compared with KRAS wt/BRAF wt (N = 109), those with KRAS codon 13 mutations (N = had significantly worse OS (P = 0.016; multivariate HR = 2.7, P = 0.011), whereas KRAS codon 12-mutated cases were not significantly associated with survival (P < 0.001), more distant metastases (P = 0.025), and worse overall survival (OS, P < 0.001; multivariate HR = 4.2, P = 0.004) in colon cancer patients. They concluded that among the three most common KRAS mutations, G13D showed significant association with poor OS [P =0.0024; multivariate HR = 2.6, P = 0.016) compared with KRAS wt/BRAF wt patients. Furthermore, $BRAF^{V600E}$ has been shown to be an independent prognostic factor for OS, next to sex and TNM, in Chinese patients.
Similar results were reported by Won, *et al.* [60], that identified 1096 patients who underwent surgery for CRC in their Hospital.
After the analysis of the CRC specimens it has been demonstrated the value of BRAF as negative prognostic factor. In fact, patient with BRAF mutations had a worse DFS (HR = 1.990, CI 1.080–3.660, P = 0.02) and OS (HR = 3.470, CI = 1.900–6.330, P < 0.0001).
Interestingly, no significant differences were found regarding KRAS as well as MSS/MSI-L and MSH.
The authors concluded highlighting the role of BRAF as a significant prognostic factor in Korean CRC patients at both early and advanced stages.
Taieb and co-workers conducted a prospective study collecting biospecimens from stage III colon cancer [53]. Morever, they performed separate analysis of MSI and MSS tumors from patients receiving adjuvant FOLFOX +/- cetuximab in two adjuvant therapy trials.
They showed that in MSS patients, all $BRAF^{V600E}$ mutations (HR = 1,54, 95% CI= 1,23 to 1,92) and KRAS codon 12 alterations (HR = 1,60, 95% CI = 1,40 to 1,83, P< 0,001) were associated whit shorter time to recurrence (TTR) and shorter survival after relapse (SAR) (HR = 3.02, 95% CI= 2.32 to 3,93, P<0,001, and HR= 1,20, 95% CI 1,01 to 1.44 P= 0,04 respectively). OS in MSS patients was poorer for BRAF-mutant patients (HR= 1,20, 95% CI= 1.56 to 2,57, P < 0.001 and KRAS-mutant patients (HR= 1.62, 95% CI = 1.38 to 1,91, P< 0,001) vs wild-type. No prognostic role of KRAS or BRAF mutations was seen in MSI patients.
The prognostic effect of BRAF and KRAS mutations compared to double wild-type cancers (dWT) has rarely been studied in MSI cancers. In fact, except for one study [63], previous reports have not found KRAS mutation status to have prognostic impact in the MSI subset of CRC. Few results have been described for BRAF [64].
De Cuba, *et al.* [61], due to the strong correlation of BRAF mutation with age, used Cancer Specific Survival (CSS) to compare BRAF and KRAS mutated cancers with dWT. This approach is the most advantageous considering the same oncogenic pathway.
They demonstrated the prognostic importance of BRAF and KRAS mutations in stage II and III colon cancers. In fact, in univariate analysis, mutations in BRAF or KRAS if compared to dWT cancers (i.e., BRAF and KRAS wild-type) (5-year CSS 93%, 95% CI 84–100%) were associated with a worse 5-year CSS of respectively 76% (95% CI 66–86%) and 77% (95% CI 59–95%), also showing similar HR of 3.61 (95% CI 1.05–12.39) and 3.46 (95% CI 0.83–14.49), respectively. For KRAS this was not statistically significant. This data is due to the low frequency of KRAS mutations in MSI colon cancers. Tumor stage was a prognostic factor demonstrating a worse CSS for stage II compared to stage II (HR = 2.62, 95% CI = 1.14-5.98; P = 0.02).
At last, after stratifying for stage, cancers with either BRAF or KRAS mutation status were associated with worse CSS in stage II, but not in stage III.
The results of multivariate analysis were consistent with those previously described.
Considering the lack of data about the role of KRAS and BRAF in stage II colon cancer alone Li Li and colleagues [57] analysed the mutation status of four key genes (KRAS, NRAS, BRAF and PIK3CA) and the incidence of dMMR in stage II CRC patients without chemotherapy after radical surgery.
Among 160 patients with stage II (Rectum 82/160, 51.3%; Descending colon 35/160 21.9%; Ascending colon 27/160 16.9%; Transverse Colon 16/160 10%) KRAS mutations were identified in 45.6% (73/160) of samples while BRAF mutations in exon 15 were detected in ten patients (6.3%, 10/160). KRAS mutation status was not associated with OS (P = 0.372). The mortality rate of patients with KRAS mutation was 17.8% (13/73), while that of patients KRAS wt was 13.8% (12/87). They found no significant correlation between the specific exons 2 and 3 KRAS mutations and the PFS (P = 0.274) and OS (P = 0.658). In patients with BRAF wt, the incidence of recurrence/metastasis and mortality was 37.3% (56/150) and 13.3% (20/150), respectively. Furthermore, BRAF mutation status significantly correlated with PFS (P = 0.008) and OS (P = 0.004).
These results, according to the authors, suggest that combinations of mutated KRAS and BRAF in stage II CRC can provide useful prognostic information beyond evaluation of either variable alone.
Dienstmann, *et al.* [47] confirmed the previous study, showing that there is a statistically significant increase in the performance of models when KRAS/BRAF mutation and MSI status are added to TNM models. In particular, this combination improves the ability to discriminate risk of death over TNM staging alone in stage II and III CRC. Unfortunately, there were no consistent improvement in

the prediction accuracy in multivariable models that include clinic-pathological features, particularly in chemotherapy-treated patients.

Less is known about the prognosis of pMMR CRC with $BRAF^{V600E}$ or KRAS mutations arising via a serrated pathway [65].

In a recent study conducted by Sinicrope el al [50], 2720 patients with stage III colon cancers were treated with the current standard adjuvant FOLFOX regimen and classified in 5 subtypes using a combination of $BRAF^{V600E}$, KRAS mutations, MLH1 methylation, and MMR status.

They found that a significantly lower proportion of patients with pMMR tumors with $BRAF^{V600E}$ (hr = 1.43, 95% CI = 1.11–1.85, Padjusted = .0065) or mutant KRAS (HR = 1.48; 95% CI 1.27– 1.74, Padjusted < .0001) survived disease free for 5 years compared to patients whose pMMR tumors lacked mutations in either gene.

DFS of patients with dMMR sporadic or familial subtypes did not differ from patients with pMMR tumors without $BRAF^{V600E}$ or KRAS mutations.

This biomarkers-based classification can provide important prognostic information in stage III colon cancer changing patient management.

At present, no evidence exists that KRAS mutations are independent prognostic factors in CRC and several studies have failed to demonstrate a correlation [66,67].

Conversely, the RASCAL studies (I and II) indicated that only one specific KRAS mutation (the glycine to valine substitution in codon 12, G12V), was associated with poorer outcome in patients with Dukes' C disease [68,69]. Kadowaki, *et al.* [52] conducted the largest study trying to assess the prognostic value of KRAS and BRAF mutations for survival outcomes in CRC patients in Asian population. They collected prospectively 813 tumor specimens of stage I-III CRC. The % of KRAS and BRAF mutations were consistent with literature [312/812 (38%) and 40/811 (5%) tumors, respectively].

KRAS mutations were associated with poorer DFS (HR = 1.35; 95% CI = 1.03-1.75) and OS (HR = 1.46; 95% CI = 1.09-1.97) and BRAF mutations were poor prognostic factors for DFS (HR = 2.20; 95% CI = 1.19-4.06) and OS (HR = 2.30; 95% CI = 1.15-4.71). Furthermore, they found that survival outcomes in Japanese CRC patients with KRAS or BRAF mutations are independent from MSI status.

A French prospective multicenter study [54] was conducted in 251/256 non-metastatic adenocarcinoma (stage I-III) of the colon with complete surgical resection.

They investigated several proteins that are targeted by therapies approved in CRC as well as markers involved in fluoropyrimidine pharmacology, such as TS (Thymidylate synthase, main 5FU target) and TP (Thymidine phosphorylase, pyrimidine anabolism), reporting that the only significant predictor of relapse-free survival (RFS) was tumor staging.

This was the first study revealing that dMMR tumours significantly exhibit elevated protein expression for TS and TP, whereas pMMR tumours express low levels of these markers. Analyses restricted to stage III showed a trend towards a shorter RFS in KRAS-mutated (P = 0.005), BRAF wt (P = 0.009) tumors and a weak tendency towards a longer RFS in dMMR tumours (P = 0.036).

Zlobec, *et al.* [59] confirmed that BRAF mutation was a poor prognostic indicator in patients with CRC. Interestingly, results showed that in patients with right-sided disease (P = 0.01) BRAF mutation appears to be an independent prognostic factor not only of pT and pN classification but also considering vascular invasion and MSI status.

Few studies have evaluated BRAF and KRAS individually.

In particular, Lochhead, *et al.* [48], using the database of two nationwide prospective cohort studies, tested the hypothesis that BRAF/MSI status could be useful as a prognostic molecular marker. MSS/BRAF-mutant tumors were associated with the highest mortality meanwhile patients with MSI-high/BRAFwt tumors experienced the lowest mortality. Authors revealed that compared with the majority subtype of MSS/BRAF wt, MSS/BRAF-mutant, MSI high/BRAF-mutant and MSI-high/BRAF wt subtypes showed multivariable colorectal cancer-specific mortality hazard ratios of 1.60 (95% CI =1.12 to 2.28; P = 0.009), 0.48 (95% CI = 0.27 to 0.87; P = 0.02), and 0.25 (95% CI = 0.12 to 0.52; P < .001), respectively.

Discrepancies regarding the prognostic value of KRAS mutations may be due to the heterogeneity of the studies populations, the influence of primary tumor site, tumor stage, and adjuvant treatment received.

Three studies investigate only KRAS mutation status. In the first study (i.e. PETACC-3 trial) Blons, *et al.* [55] showed that when specific mutations were compared with wild-type, KRAS codon 12 mutations [HR 1.67, 95% CI = 1.35–2.04, P < 0.001] but not codon 13 (HR 1.23, 95% CI 0.85–1.79, P = 0.26) were significantly associated with shorter TTR, independently of other covariates. Subgroup analysis showed that KRAS only affected TTR and DFS in distal tumors (n = 1043; 692 wild type; 351 mutated), with an increased risk of relapse (HR 1.96, 95% CI 1.51–2.56, P < 0.0001) for KRAS codon 12 mutations and a borderline significance for codon13 mutations (HR 1.59, 95% CI 1.00–2.56, P = 0.051).

The authors failed to demonstrate any association between KRAS mutation and relapse or survival among the 1404 colon cancer patients treated with 5-FU +/- irinotecan.

Secondly, Ogino, et al. [49] examined the influence of KRAS in 508 patients stage III CRC being part of the National Cancer Institute-sponsored Cancer and Leukemia Group B (CALGB).

In this study KRAS mutational status was not associated with any significant influence on cancer recurrence or death. These results were confirmed in multivariate analysis.

Lastly, Shen, *et al.* [58] showed no significant differences in DFS between patients with KRAS wt and mutants (P = 0.727). Furthermore subgroups such as codon 12/13 and mutation types (G12D/G12V/ G12C/G12S/G13D) did not appear as prognostic factors for DFS However, *KRAS* codon 61 mutation was prognostic for DFS in stage III alone, but not in stage II or the whole population. KRAS mutation status was not prognostic for OS, no matter analyzed together or separately.
Studies that investigated the role of MMR status shows that was not significantly associated with the time-to-event variables (DFS and OS).
Sasaki, et al. [51] found that KRAS/NRAS mutations were significantly associated with the benefit of adjuvant chemotherapy with tegafur-uracil (UFT) in relapse-free survival (RFS) (HR = 0.49; P = 0.02) and OS (HR = 0.51; P = 0.03). In contrast, among patients with non-mutated KRAS/NRAS there was no difference in RFS or OS between the adjuvant UFT group and surgery-alone group. The MMR status was neither prognostic nor predictive for adjuvant chemotherapy.
Finally, Yoon, *et al.* [46] tried to assess racial disparities (Asian, black or white) selecting several well-described randomized trials.
KRAS mutation rates were highest in tumors from blacks (44.1%). Conversely, tumors with KRAS-wt/BRAF-wt mutations were most common among Asians (66.7%).
Compared with whites, blacks had shorter DFS and TTR among patients younger than age 50 years or with N1 disease, independent of BRAF, KRAS, and other covariates.
Considering N2 tumors Asians had longer DFS for the lower expression of BRAF mutation.
Few data are available regarding the role of KRAS and BRAF mutations in Asian populations.
In fact, most reports are from Western Countries. For this reason, the major strength of our study, in addition to the large sample size, was utilizing data from different geographical areas (USA, Europe and Asian countries).
Nevertheless, the two major limitations, represented by the retrospective nature of most studies and the heterogeneity cohort of patients, must be taken into account.

Conclusions

CRC is a global public health problem with a complex aetiology. Surgical resection offers a potential cure for patients with CRC only if diagnosis is performed with optimal timing.
In our opinion, a combination of molecular markers, tumor location with the other clinical-pathological variables and microsatellite status is essential to have a correct prognosis. Nevertheless, this combination could be useful as a predictive factor in stage I-III CRC.

Riassunto

Il carcinoma colon-rettale (CRC) è una delle neoplasie più frequenti per incidenza e mortalità nei
Paesi industrializzati con circa 1.2 milioni di nuovi casi ogni anno.
Le alterazioni genetiche ed epigenetiche caratterizzano il processo di carcinogenesi del CRC e sono essenziali per l'identificazione e lo sviluppo di un biomarker ideale.
Nel primo caso, i due più importanti pathways sono rappresentati dal pathway di EGFR (Epidermal Grow Factor Receptor), con il coinvolgimento di KRAS e BRAF, e dal sistema del DNA mismatch repair (MMR).
Le alterazioni epigenetiche, invece, influenzano il fenotipo senza alterare il genotipo ed includono l'espressione dei microRNA (miRNAs), i processi di silenziamento genico e di metilazione del DNA.
Attualmente, il tasso di sopravvivenza a 5 anni (OS) dei pazienti con CRC localizzato arriva fino all'80-90%, mentre per i pazienti con tumore localmente avanzato non metastatico o metastatico arriva, rispettivamente, al 40-60% ed al 5-10%. Questo a causa di una incompleta compresione dei meccanismi molecolari che regolano la patogenesi, l'alto tasso di recidive e lo sviluppo di resistenza alle target-therapy.
Lo scopo di questa systematic review è di valutare l'impatto ed il significato prognostico delle mutazioni di BRAF/KRAS in pazienti con CRC I-III stadio.
Dopo una prima ricerca nei 4 principali databases (Pubmed, Embase, Web Of Science And Cochrane Library) sono stati identificati 92 studi.
La successiva valutazione dei criteri di inclusioni oltre che delle caratteristiche e della qualità degli studi ha consentito di includere 16 articoli in questa systematic review.
I risultati di questo studio mettono in evidenza la necessità di un'associazione fra markers molecolari tumorali, caratteristiche clinico-patologiche del tumore e status dei microsatelliti ai fini di avere una corretta prognosi dei pazienti con CRC I-III stadio.
La maggior parte dei dati su BRAF/KRAS presenti in letteratura non includono l'identificazione del loro ruolo nelle popolazioni Asiatiche.
Per questo motivo, il maggior potere di questa systematic review è stato l'utilizzo di dati provenienti da differenti aree geografiche (USA, Europa e Asia).
Tuttavia, l'eterogeneità dei dati e la natura retrospettiva della maggior parte degli studi inclusi rappresentano i due maggior limiti.

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All authors equally contributed to this work satisfying

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Gaetano Gallo and Giuseppe Sena: contributed equally to this work: substantial contributions to the conception and design of the work; acquisition, analysis, and interpretation of data for the work; drafting of the work and revising it critically for important intellectual content; final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of any part of the work are appropriately investigated and resolved.

Giuseppina Vescio, Matteo Papandrea, Rosario Sacco, Mario Trompetto and Giuseppe Sammarco: contributed equally to this work: final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of any part of the work are appropriately investigated and resolved.

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