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SPECIAL ARTICLE

Guidelines for biomarker testing in colorectal carcinoma (CRC): a national consensus of the Spanish Society of Pathology (SEAP) and the Spanish Society of Medical Oncology (SEOM)

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Abstract This consensus statement, conceived as a joint initiative of the Spanish Society of Pathology and the Spanish Society of Medical Oncology, makes diagnostic and treatment recommendations for the management of patients with hereditary, localised and advanced CRC based on the current scientific evidence on biomarker use. This consensus statement thus provides an opportunity to improve healthcare efficiency and resource use, which will benefit these patients. Based on the currently available data on this subject, this expert group recommends testing for microsatellite instability (MSI) in patients with localised CRC, as this is a strong predictive factor for deciding on adjuvant treatment. However, although the ColoPrint® and Oncotype Dx® gene expression signatures have been shown to have prognostic value, no consensus yet exists concerning their use in clinical practice. For advanced CRC, it is essential to test for *KRAS* mutation status before administering an anti-EGFR treatment, such as cetuximab or panitumumab. However, testing for other biomarkers, such as *BRAF*, *EGFR*, *PI3K* and *PTEN* mutations, should not be done routinely, because this does not influence treatment planning at the present time. Other important issues addressed include organisational requirements and the quality controls needed for proper testing of these biomarkers as well as the legal implications to be borne in mind when testing some biomarkers.

Keywords EGFR · KRAS · BRAF · MSI · PTEN

Introduction

Recently, the health sector has encouraged the writing of clinical practice guidelines, which has resulted in more

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effective oncological intervention based on scientific evidence levels and professional consensus. In this respect, no specific clinical guidelines exist on the use of biomarkers in colorectal cancer (CRC).

The high incidence of CRC, and its impact on public health mean that consensus protocols need to be drawn up by the multidisciplinary teams involved in designing the various treatment strategies. In order to work together with the basic aim of curing patients, or at least improving their survival and quality of life, oncologists and pathologists must communicate with each other fluently and consistently. On the other hand, using these consensus protocols will be financially advantageous, as it will optimise administration of the most appropriate treatments in each case.

One of the revolutions in treatment currently taking place relies on understanding the biomarkers involved in molecular pathways in cancer cells. Drugs designed to block some of these have been incorporated into the therapeutic arsenal available. Other biomarkers, such as genes involved in the deoxyribonucleic acid (DNA) repair pathway or the *APC* gene, are already routinely tested to determine the subject's susceptibility to hereditary CRC. These new treatment strategies require oncologists and pathologists to work together, to ensure that the necessary specimen is available to enable these markers to be tested in the relevant laboratories.

Although in most cases material from surgically resected CRC provides a guaranteed source of sufficient sample, it must be remembered that under certain circumstances this material may be limited and in short supply. This is especially true when only endoscopic biopsies or needle biopsies of metastatic disease are available, or even when only minimal residual tumour remains after neoadjuvant treatment. For all these reasons, the pathologist must take responsibility for optimising the use of specimens, and their traceability in the event that biomarker tests take place in different laboratories.

One key issue covered in these guidelines is the optimisation and standardisation of methodology used for biomarker testing. Also, in order to ensure the effectiveness and specificity of the results, internal or external quality controls must be applied, such as those implemented by scientific associations such as the Spanish Society of Medical Oncology (SEOM) and the Spanish Society of Pathology (SEAP).

Another important subject addressed in these guidelines concerns the legal implications and bioethical issues surrounding the management of cancer patients. Observation of the new Biomedical Research Act requires ethical aspects to be considered, such as obtaining informed consent and regulations about storing surplus specimens in various biobanks. Obtaining informed consent is crucial, as some genetic tests, such as microsatellite instability (MSI), may provide information that has repercussions for the patient's family, so the patient may refuse to have them done.

These guidelines demonstrate the degree of consensus that exists between pathologists and oncologists, and aim to establish clearly and concisely when tests need to be done for the various markers of hereditary susceptibility to CRC, as well as other prognostic and predictive biomarkers. These guidelines may also be revised from time to time, to incorporate further biomarkers when the level of scientific evidence makes their use advisable, either in the context of clinical trials or in future treatment strategies.

Clinical issues

Markers of susceptibility to hereditary CRC

About 26,000 cases of CRC are diagnosed every year in Spain, of which 5–10 % display a clearly hereditary pattern and up to 25 % have a familial pattern. Identifying these cases is enormously important in order to increase the rate of early diagnosis. The fact that early diagnosis of CRC can achieve cure rates of up to 90 %, similar to rates obtained with breast screening, speaks for itself.

Patients with hereditary forms of CRC can be divided into those with Lynch syndrome, also called hereditary nonpolyposis colon cancer, and those with polyposis syndrome. These in turn are subdivided into patients with adenomatous and non-adenomatous forms.

Lynch syndrome

When a patient with Lynch syndrome is identified by means of the Amsterdam I and Amsterdam II clinical suspicion criteria [1, 2], he or she should be offered the chance to be tested for mutations in the mismatch repair (*MMR*) gene family, basically *MLH1*, *MSH2*, *MSH6* and *PMS2*, as part of appropriate genetic counselling [3]. This should be done by following a clear, concise action algorithm, agreed between the groups working in this field, such as the one shown in Fig. 1. Thus, the first step in a patient who meets the clinical criteria for suspicion should be to test for MSI or non-expression of the protein products of these genes, by means of immunohistochemistry (IHC). Depending on the presence or absence of mutations in the *MMR* genes, more specific guidance can be given regarding the risk of carriers developing one of the cancers associated with Lynch syndrome, as well as how best to monitor them in terms of tests, regularity and age of onset [3].

Microsatellite instability testing may also be recommended in some cases of non-familial CRC, as up to 10 %

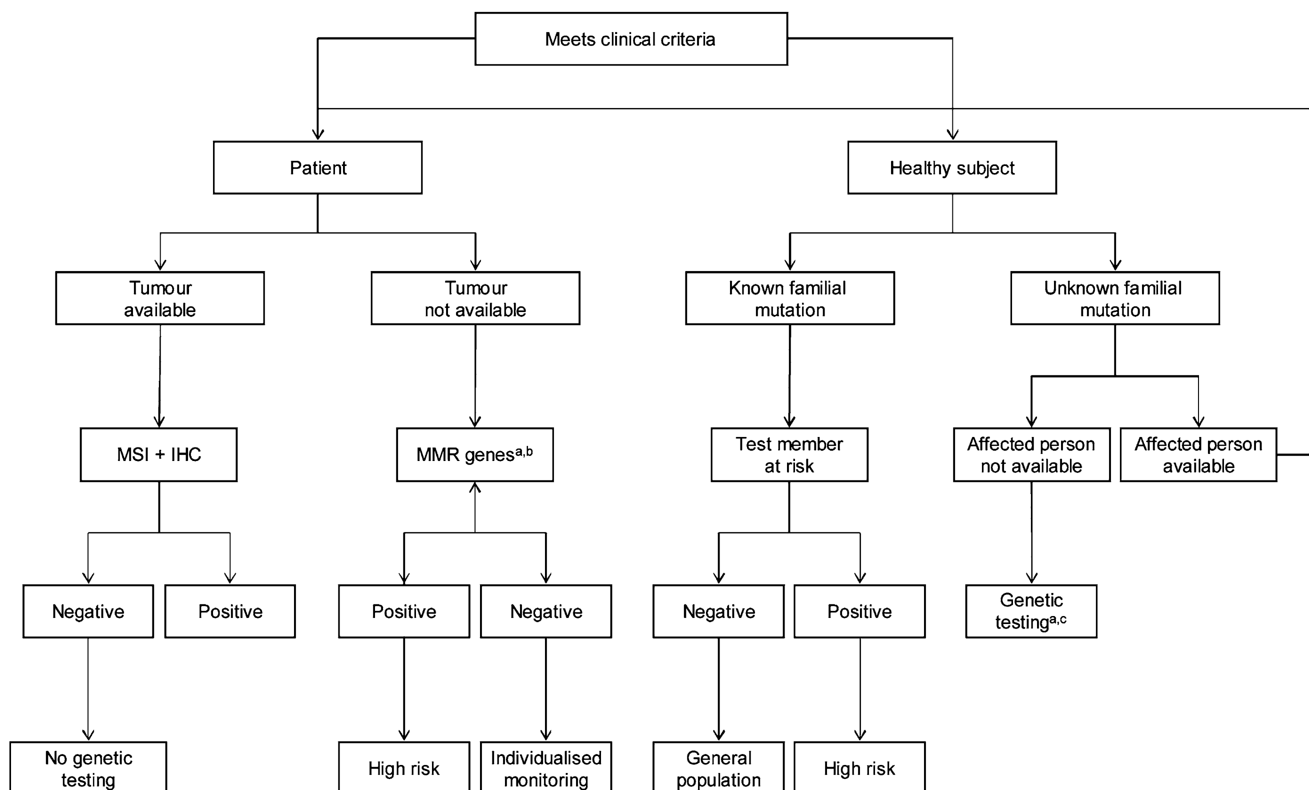

Fig. 1 Action algorithm to detect Lynch syndrome. [a]Assess individually. [b]When no tumour is available, but the Amsterdam criteria are met and there are major implications for the family, the option of directly testing for germ-line mutations in MMR genes should be considered. [c]When there are no affected individuals alive or available, germ-line testing in a healthy subject should be considered, provided there are major repercussions for management of the family. *IHC* immunohistochemistry, *MMR* post-replication mismatch repair genes, *MSI* microsatellite instability

of these CRCs may express it [4]. Moreover, it has recently been suggested that the indications for this type of testing should be extended to any CRC or endometrial cancer, as it has proved cost-effective, at least in CRC. Finally, there are mathematical models that can help determine the likelihood of a family having a mutation in the *MMR* genes [5].

Polyposis syndromes

Table 1 lists the genes associated with both polyposis and nonpolyposis syndromes. To date, no molecular markers exist to enable identification of polyposis cases. Families are, therefore, initially selected by exclusively clinical criteria and the genes implicated in each are subsequently tested for germ-line mutations. It must be remembered that up to 30 % of cases of familial adenomatous polyposis involve only the index case, with no other family history. In any event, this fact should make no difference when it comes to suggesting a genetic study.

Based on the above, this expert panel recommends that:

- To detect markers of susceptibility to hereditary CRC, relatives of patients with CRC should be pre-screened, bearing in mind the Amsterdam and Bethesda clinical suspicion criteria (Level of Evidence IIa).
- If there is no MSI, and no loss of expression of any repair protein, genetic testing of the patient should proceed no further, as a pathogenic mutation in the repair genes is unlikely to be identified (Level of Evidence IIa).

Molecular markers of localised CRC

CRC tumorigenesis is driven by the accumulation of a limited number of genetic alterations in oncogenes and tumour suppressor genes. These are well understood, but applying them clinically has proved elusive until recently. A lack of standardised validation procedures for new prognostic and predictive molecular biomarkers may partly explain this slow transition into the clinic.

18q loss of heterozygosity

There are studies suggesting that 18q loss of heterozygosity (LOH) is a prognostic biomarker [6], loss of expression of

Table 1 Hereditary CRC syndromes and associated genes

Syndrome	Gene
Lynch syndrome	*MLH1*
	MSH2
	MSH6
	PMS2
	EPCAM
Familial adenomatous polyposis	*APC*
	MYH
Juvenile polyposis	*SMAD4*
	BMP[a]
Cowden syndrome	*PTEN*
Peutz–Jeghers syndrome	*STK11*

CRC colorectal cancer

These genetic abnormalities are determined at the germ-line level

DCC and SMAD4 being the putative molecular targets involved [7, 8]. However, data obtained in the most robust study to address this question within the randomised, prospective PETACC-3 trial failed to validate this prognostic biomarker when the analysis was controlled by MSI status, either at stage II or at stage III [9]. Similarly, a study conducted on a cohort of 555 microsatellite stable tumours, including stages I–IV, ruled out any prognostic value for 18q LOH [10].

MSI

In contrast, the prognostic value of MSI, initially also suggested by a meta-analysis of retrospective and heterogeneous studies [11], has been validated in various analyses associated with large prospective phase III trials [9, 12–14]. Results from the PETACC-3 study validate this effect at stage II but not at stage III [hazard ratio (HR) 0.28; 95 % confidence interval (CI) 0.1–0.72; $p = 0.0089$) [9]. In ancillary analysis in the QUASAR study, MSI evaluated by repair protein IHC was the strongest prognostic risk factor for recurrence (HR 0.53; 95 % CI 0.40–0.70; $p < 0.001$) [13].

Consequently, although the potential of MSI as a negative predictive marker of benefit from adjuvant chemotherapy is controversial [15], MSI is the only prognostic biomarker that has been sufficiently validated in independent and prospective studies, in multivariate analysis including other relevant clinicopathological risk factors.

Gene expression signatures

Genetic changes detected in CRC can lead to global changes in the transcriptome that have been extensively confirmed by high-throughput techniques.

Several studies have examined the prognostic value of expression profiles, yielding promising results [16–24]. However, these studies have important limitations, because: (1) a small number of samples are used to generate the gene expression signatures; (2) there is no independent external cohort of samples to validate the findings obtained; and (3) no comparisons are available between these molecular classifiers and standard clinicopathological factors used in clinical practice.

Nevertheless, two commercial platforms have overcome these limitations. One of these, the Oncotype Dx® colon cancer assay, is already commercially available in the United States. It was initially developed by randomly testing 761 candidate genes from samples obtained from patients with stage II and III colon cancer enrolled in the clinical trials NSABP C-01/C-02/C-04/C-06 and CCF, using reverse transcription-polymerase chain reaction (RT-PCR) techniques [25]. In this way, two sets of seven and six genes were selected for validation as prognostic and predictive signatures, respectively. This was done by prospectively testing 1,200 samples from patients included in the QUASAR trial and correlating the results with patient outcomes. This enabled the prognostic value of the gene signature to be validated, establishing three risk categories (high, intermediate and low) with relapse rates at 3 years of 22, 18 and 12 % ($p = 0.046$), respectively. However, the predictive value of the gene signature could not be validated, which compromises the clinical usefulness of this test [26].

The other commercial platform, ColoPrint®, was developed as a prognostic assay on 44K Agilent multigene microarrays of complementary deoxyribonucleic acid (cDNA), using fresh tumour samples and an initial agnostic approach. The most robust 18-gene expression signature thus created classifies patients as having a low versus high risk of relapse, independently of clinicopathological factors [27]. This platform was recently validated in an independent series, in which multivariate analysis demonstrated that ColoPrint® has prognostic value for relapse-free survival (RFS) (HR 2.69; 95 % CI 1.41–5.14; $p = 0.003$), especially in stage II patients (HR 3.34; $p = 0.017$) [28]. Another independent series has reproduced similar results [29], while the prospective confirmatory study PARSC (Prospective Study for the Assessment of Recurrence Risk in Stage II Colon Cancer Patients Using ColoPrint™ NCT00903565 http://165.112.8.96/ct2/show/results/NCT00903565) is ongoing. Therefore, although results with ColoPrint® are encouraging, with an almost 20 % difference in relapse risk survival, they need to be validated in independent prospective studies before it can be recommended for use in clinical practice.

Finally, the gene signature developed by Almac Diagnostics in formalin-fixed paraffin-embedded (FFPE)

tumour specimens has recently been validated in an independent retrospective patient series. However, the study does not describe MSI status. This is a major concern, as it is impossible to estimate the relative contribution of this marker to the performance of this new gene signature [30].

In summary, although many individual molecular biomarkers have been developed for localised CRC, and gene expression signatures have yielded promising results, at the present time only the prognostic value of MSI is sufficiently validated for it to be used in clinical practice.

Based on the above, this expert panel recommends that:

- Patients with stage II CRC and MSI should not be candidates for adjuvant chemotherapy, because they have a low risk of relapse and there is no scientific evidence that this treatment will benefit them (Level of Evidence IIa).

Molecular markers of metastatic CRC

Downstream of the epidermal growth factor receptor (EGFR), once this has been activated, signal transmission can follow three main intracellular signalling pathways: (1) via RAS–BRAF–MAPK activation; (2) via PI3K–AKT–PTEN–mTOR activation; and (3) via the signal transducer and activator of transcription 3 (STAT3) pathway. Various drivers of the proliferative signal thus exist, such as KRAS, BRAF, EGFR, PI3K and PTEN.

KRAS

Three members of the *RAS* gene family are known: *H-RAS*, *N-RAS* and *KRAS*, the latter being most often mutated in CRC. Under normal conditions, these genes encode a set of RAS proteins, which transmit signalling produced by the activation of membrane receptors. The inactive RAS protein is bound to guanosine diphosphate (GDP) and, when stimulated, a guanine nucleotide exchange factor (GEF) promotes formation of the guanosine triphosphate complex (GTP)-RAS, which is the active form of the protein. In short, that GTP is hydrolysed to GDP by the activity of a GTPase intrinsic to the RAS proteins, inactivating it. However, when mutations exist in *KRAS*, GTPase activity is blocked and the RAS protein remains constitutively activated and bound to GTP. These mutations tend to occur in codons 12 and 13 and, much less often, in codon 61.

It is important to note that, although *KRAS* status has not been shown to have prognostic value in patients with stage II and III CRC, as concluded from examining a total of 1,564 samples obtained from 3,278 patients recruited to various clinical trials with adjuvant chemotherapy (PETACC-3, EORTC 40993 and SAKK 60-00) [31], in patients with metastatic CRC *KRAS* status has a decisive influence when it comes to planning the patient's treatment.

The CRYSTAL trial evaluates the benefit of adding cetuximab to the FOLFIRI regimen (folinic acid, fluorouracil and irinotecan) as first-line treatment in patients with metastatic CRC. In this study, the percentage of patients subjected to *KRAS* analysis was 89 %, of whom 37 % had *KRAS* mutations. In the *KRAS* wild-type patient group, addition of cetuximab significantly increased progression-free survival (PFS) (9.9 vs. 8.4 months; HR 0.696; $p = 0.0012$), overall survival (OS) (23.5 vs. 20.0 months; HR 0.796; $p = 0.0093$) and overall response rate (ORR) (57.3 vs. 39.7 %; HR 2.069; $p < 0.001$) [32, 33].

The OPUS (Oxaliplatin and Cetuximab in First-Line Treatment of Metastatic Colorectal Cancer) randomised phase II clinical trial evaluated *KRAS* status in 315 samples, representing 93 % of patients recruited. In 179 (57 %) of them it was not mutated. In these patients, it was observed that adding cetuximab to the FOLFOX-4 regimen (fluorouracil, leucovorin and oxaliplatin) as first-line treatment for metastatic CRC only benefited patients with wild-type *KRAS*, significantly increasing PFS (8.3 vs. 7.2 months; HR 0.567; $p = 0.0064$) and ORR (57.3 vs. 34.0 %; HR 2.551; $p = 0.0027$) compared with patients treated with FOLFOX-4 alone. However, the increase seen in OS did not attain statistical significance in these patients (22.8 vs. 18.5 months; HR 0.855; $p = 0.39$) [34, 35].

The CELIM trial included 114 patients at 17 sites in Germany and Austria who were randomised to receive cetuximab plus FOLFOX-6 (56 patients) or cetuximab plus FOLFIRI (55 patients) [36]. The primary endpoint was ORR assessed by response evaluation criteria in solid tumours (RECIST). Partial or complete response was confirmed in 68 % of patients in Group A and 57 % in Group B, with a difference of 11 % between groups (95 % CI −8 to 30; HR 1.62, 0.74–3.59; $p = 0.23$). R0 resection was achieved in 20 % of Group A and 30 % of Group B. The ORR in the *KRAS* wild-type group was 70 versus 41 % in *KRAS*-mutated patients (HR 3.42, 1.35–8.66; $p = 0.0080$).

The COIN randomised clinical trial compared three regimens as first-line treatments in patients with metastatic CRC [37]. Arm A was given oxaliplatin plus fluoropyrimidines (capecitabine or 5-fluorouracil infusion plus leucovorin); Arm B had the same regimen plus cetuximab; and Arm C received intermittent chemotherapy. Comparison of the results obtained in Arms A and B showed that in 1,316 patients (81 %) tested for KRAS, 565 (43 %) had mutated *KRAS*. In patients with wild-type *KRAS*, no differences in PFS were seen between Arm A [8.6 months; interquartile range (IQR) 5.0–12.5] and Arm B with cetuximab (8.6 months; IQR 5.1–13.8; HR 0.96; 95 % CI 0.82–1.12; $p = 0.60$). No differences were seen in OS either, with a median of 17.9 months (IQR 10.3–29.2) in

Arm A and 17.0 months (IQR 9.4–30.1) in Arm B (HR 1.04; 95 % CI 0.87–1.23; $p = 0.67$). In contrast, ORR did show a significant increase ($p = 0.049$) in the cetuximab arm (64 %), compared against the arm treated with chemotherapy only (57 %).

A consortium of eleven hospitals in seven European countries managed to collect 1,022 specimens (949 in paraffin blocks and 73 fresh-frozen tissue samples) from patients treated with cetuximab between 2001 and 2008 [38]. Forty per cent of the tumours (299 out of 747 samples tested) were found to carry *KRAS* mutations. The ORR was 35.8 % in patients with *KRAS* wild-type tumours, compared with 6.7 % in patients with *KRAS*-mutated tumours (HR 0.13; 95 % CI 0.07–0.22; $p < 0.0001$). The median PFS was 24 versus 12 weeks (HR 1.98; 95 % CI 1.66–2.36; $p < 0.0001$) and the median OS favoured patients without a *KRAS* mutation (50 vs. 32 weeks; HR 1.75; 95 % CI 1.47–2.09; $p < 0.0001$).

Based on patients enrolled in the CO.17, BOND, MABEL, EMR202600, EVEREST, BABEL and SALVAGE trials, *KRAS* mutations were evaluated to see if they all had the same prognostic impact [39]. Patients with the G13D mutation were found to show significantly better PFS than those with the rest of the mutations (4.0 months; 95 % CI 1.9–6.2 vs. 1.9 months; 95 % CI 1.8–2.8) with an adjusted HR of 0.51 (95 % CI 0.32–0.81; $p = 0.004$), as well as a significant OS benefit of 7.6 months (95 % CI 5.7–20.5) versus 5.7 months (95 % CI 4.9–6.8) with an adjusted HR of 0.50 (95 % CI 0.31–0.81; $p = 0.005$). Studies in cell lines and animal models show that lines carrying the G12V mutation are insensitive to cetuximab, whereas those with G13D show sensitivity similar to that of wild-type cell lines.

Finally, the phase III clinical trial PRIME (Panitumumab Randomised Trial in Combination with Chemotherapy for Metastatic Colorectal Cancer to Determine Efficacy) demonstrated, in patients with *KRAS* wild-type tumours, a statistically significant benefit in PFS for the group treated with panitumumab and FOLFOX-4 (10.0 months; 95 % CI 9.3–11.4) compared with the group treated with FOLFOX-4 alone (8.6 months; 95 % CI 7.5–9.5; HR 0.80; 95 % CI 7.5–9.5; $p = 0.009$) [40]. The ORR also reflected a significant increase in the group treated with panitumumab, with 57 % (95 % CI 51–63) versus 48 % (95 % CI 42–53) observed in the control group (HR 1.47; 95 % CI 1.07–2.04; $p = 0.018$). In terms of OS, no significant differences were seen in favour of panitumumab compared with the control arm (23.9 vs. 19.7; HR 0.83; 95 % CI 0.67–1.02; $p = 0.072$). Nevertheless, a re-evaluation of the OS results, applying sensitivity analysis by the Branson–Whitehead approach, was presented at the 2011 meeting of the American Society of Clinical Oncology (ASCO) [41]. This showed a HR value of 0.74 (95 % CI 0.61–0.90; $p = 0.003$) for *KRAS* wild-type cases. Results obtained in phase III studies conducted in patients with *KRAS*-mutated metastatic CRC are summarised in Table 2.

BRAF

The *BRAF* gene, which encodes one of the main intracellular effectors of *KRAS*, is mutated in 5–10 % of patients with CRC [38]. The most common mutation (~90 %) is the V600E substitution, located in the kinase domain of the protein (exon 15). *BRAF* mutations and *KRAS* mutations are mutually exclusive, so the former are never found in *KRAS*-mutated tumours and vice versa. They are associated with the presence of high microsatellite instability (MSI-H) induced by acquired defects in MMR enzyme function [13, 31]. In other contexts, particularly in advanced disease, the presence of *BRAF* mutations is associated with a worse prognosis [13, 31, 33, 34, 38, 42, 43].

However, its role as a predictive factor for response to anti-EGFR therapies is more controversial. Although it has been consistently observed in uncontrolled studies that patients with *KRAS* wild type, *BRAF*-mutated tumours treated with anti-EGFR have lower ORR (8.3 vs. 38 %; HR 0.15; $p = 0.0012$), PFS (8 vs. 26 weeks; HR 3.74; $p < 0.0001$) and OS (26 vs. 54 weeks, HR 3.03; $p < 0.0001$) than patients with *BRAF* wild-type tumours [38], the small amount of data available from randomised trials suggests that these worse outcomes are independent of

Table 2 Impact of anti-EGFR antibody treatment in patients with *KRAS* wild-type metastatic CRC

	ORR AB + CT vs. CT	PFS AB + CT vs. CT	OS AB + CT vs. CT
CRYSTAL [33]	57.3 vs. 39.7 %; HR 2.069; $p < 0.001$	9.9 vs. 8.4 months; HR 0.696; $p = 0.0012$	23.5 vs. 20.0 months; HR 0.796; $p = 0.0093$
OPUS [34]	57.3 vs. 34.0 %; HR 2.551; $p = 0.0027$	8.3 vs. 7.2 months; HR 0.567; $p = 0.0064$	22.8 vs. 18.5 months; HR 0.855; $p = 0.39$
COIN [37]	64 vs. 57 %; $p = 0.049$	8.6 vs. 8.6 months; HR 0.96; $p = 0.60$	17.9 vs. 17.0 months; HR 1.04; $p = 0.67$
PRIME [40]	57 vs. 48 %; HR 1.47; $p = 0.018$	10.0 vs. 8.6 months; HR 0.80; $p = 0.009$	23.9 vs. 19.7 months; HR 0.88; $p = 0.072$

AB antibody, *CT* chemotherapy, *HR* hazard ratio, *ORR* overall response rate, *OS* overall survival, *PFS* progression-free survival

the treatment received [33, 34, 42]. In the CRYSTAL study, in which patients with metastatic CRC were randomly assigned FOLFIRI ± cetuximab, ORR, PFS and OS increased significantly when cetuximab was added to conventional chemotherapy in *KRAS* and *BRAF* wild-type patients ($n = 566$) [33]. However, a similar but smaller trend, which did not attain statistical significance, was seen in *KRAS* wild-type, *BRAF*-mutated patients ($n = 59$) (ORR 19.2 vs. 15.2 %; PFS 8.0 vs. 5.6 months; and OS 14.1 vs. 10.3 months in patients treated with FOLFIRI ± cetuximab, respectively). Although the small sample size prevents any definite conclusions being drawn, the presence of *BRAF* mutations appears to be of more prognostic than predictive value. Similar results were seen in the CAIRO2 study, in which the presence of *BRAF* mutations was significantly associated with PFS and OS, both in patients treated with cetuximab and in those not given that antibody [42].

EGFR

Initially, anti-EGFR therapies were developed exclusively in tumours expressing EGFR on the cell surface, as detected by IHC techniques (85 % of CRCs). It was subsequently observed, however, that there was no good correlation between EGFR protein expression and response to these drugs. In fact, ORRs of up to 25 % have been documented in *EGFR*-negative tumours. On the other hand, unlike with other cancers, the existence of *EGFR*-activating mutations is rare in CRC.

Other factors that have been suggested as potential predictive biomarkers of response to anti-EGFR therapies include over-expression of the receptor's natural ligands, such as epiregulin and amphiregulin [44], certain ligand polymorphisms (EGF61A/G vs. EGF61A/A or EGF61G/G) [45], amplification of the *EGFR* gene or chromosome 7 polysomy (both of which are associated with increased copy number of the gene) [46]. Likewise, polymorphisms in the Fc gamma receptors (FcγR) of immune effector cells (FcγRIIa-131H/H and/or FcγRIIIa-158V/V) have also been found to be associated with a better response to cetuximab-based treatment, even in tumours harbouring *KRAS* mutations. This seems to support the hypothesis that antibody-dependent cellular cytotoxicity (ADCC) might play an important role in this context. However, much of this data comes from small retrospective series, without proper controls, so no definite conclusions can be drawn concerning their clinical usefulness.

Other mutations

The PI3K–AKT–mTOR pathway is another major intracellular signalling effector pathway activated by EGFR stimulation. *PIK3CA*-activating mutations have been described in approximately 15 % of colon carcinomas, mainly in exon 9 (69 %) and exon 20 (20 %) [38]. Only mutations in exon 20 seem to be associated with worse clinical outcomes in uncontrolled series of patients treated with cetuximab, with ORR of 0 versus 37 %, PFS of 11.5 versus 24 weeks and OS of 34 versus 51 weeks in patients with mutated (exon 20) versus wild-type *PIK3CA*, respectively [38]. However, these data should be confirmed by means of prospective studies properly designed for that purpose.

On the other hand, *PTEN* is a tumour suppressor gene that inhibits the PI3K/AKT signalling pathway. Loss of *PTEN* function due to mutations, deletions or epigenetic silencing leads to activation of this pathway. Some studies have noted a significant association between loss of *PTEN* expression and less response to anti-EGFR treatments [47, 48]. However, other authors have obtained contradictory conclusions. These inconsistent results may be due either to a lack of standardisation of the *PTEN* detection technique used, or to the complexity of intracellular interactions with other pathways capable of modulating this response.

Based on the above, this expert panel recommends that:

- All patients with metastatic CRC being considered for anti-EGFR therapy should be tested for *KRAS* status, as this therapy should only be given when no mutations exist in this gene (Level of Evidence Ia).
- These patients should not be tested routinely for *BRAF*, *EGFR*, *PI3K* and *PTEN* status, as this is not necessary for therapeutic decision-making (Level of Evidence IIb for *BRAF*; Level of Evidence IIIc for *EGFR*, *PI3K* and *PTEN*).

Pathology issues

Referral centres

The study of biomarkers of susceptibility to hereditary CRC, and biological factors influencing the predicted response to therapy given or the choice of a specific treatment design, requires the participation of laboratories of recognised experience accredited by external and internal quality controls.

Ideally, the same pathology laboratory responsible for safekeeping of the specimen would perform the relevant IHC or molecular techniques. If this is not feasible, the existence of molecular biology laboratories attached to the hospital's own foundations, oncology departments or central laboratory might offer another solution [49]. After that, the results of the molecular studies will require to be integrated into the final diagnosis of the patient, together

with the appropriate morphological, immunohistochemical and molecular tests.

Accurate histological diagnosis as the first biomarker

The first histological diagnosis of a CRC comes from examining endoscopic biopsies obtained by colonoscopy, whereas the established diagnosis is based on examination of the surgical resection specimen. In order to guarantee histological diagnosis of CRC with the endoscopic material, it is essential for the tumour to be sampled properly, and for there to be at least three representative tumour samples. Very superficial biopsies of the lesion enable observation of cytological atypia and the complexity of glandular architecture, which is enough to recognise a malignant epithelial tumour. However, in order for a diagnosis of infiltrating adenocarcinoma to be made, the presence of cancerous glands within a desmoplastic stroma has to be seen, which requires biopsies of adequate size. Also, it is sometimes advisable to do molecular tests on endoscopic tumour material, which is another reason for obtaining a suitable minimum amount of biopsied tumour.

Finally, it must be remembered that CRC is not a single entity. As well as conventional adenocarcinoma, there is a whole range of primary malignancies of assorted lineages (epithelial, stromal or neuroendocrine) that must be included in the differential diagnosis according to the World Health Organisation (WHO) classification. In these cases, the use of IHC techniques can reveal the tumor's cell type, offering the chance to make an established diagnosis, provided enough endoscopic material is available [50].

From the time a tumour tissue specimen arrives in the pathology department until the results are reported, all the processes carried out can be divided into three phases, namely: (1) the pre-test phase, which includes fixing and processing the specimen, but also establishing whether a mutation study is indicated; (2) the test phase, which includes selecting the sample and the most suitable molecular technique with its corresponding controls; and (3) the post-test phase, involving interpretation of the results and issuing a molecular diagnostics report.

All these stages are essential, and problems arising in any of them can interfere with the quality of the test. The laboratory carrying out the test should be in control of all those processes and, if possible, conduct it in an integrated manner.

Pre-test phase

Once taken, endoscopic specimens should be fixed in 10 % buffered formaldehyde, for not more than 24 h, before being embedded in paraffin (Table 3).

It is advisable for tumour colectomy specimens to be sent fresh to the pathology department as soon as possible after removal, preferably within 30 min. This ensures the integrity of nucleic acids, preventing their degradation by ribonuclease (RNase) enzymes. Tumour and non-tumour tissue should then be cryopreserved for inclusion in the tumour bank and to enable the necessary molecular studies to be done, ensuring correct handling of the surgical specimen at all times. The tumour is fixed in 10 % buffered formaldehyde for 24–48 h, then cut into representative sections and embedded in paraffin. It is also advisable for a sample of healthy colonic mucosa to be preserved in paraffin, as this allows histological diagnosis of precursor lesions, and means that paraffin-embedded non-tumour tissue material is available from the patient. The representative tumour sections should also contain adjacent healthy mucosa, to enable detection of any pre-existing polyp on which the carcinoma may have developed, as well as tissue from the zone of maximum local tumour infiltration.

Carcinomas are unique lesions and any material not preserved, by being frozen or embedded in paraffin, will be lost. It is, therefore, worth remembering this and trying to preserve not less than five blocks of tumour and, if possible, allocate a sample of tumour tissue and one of healthy tissue in paraffin to the tumour bank. It is also important for healthy mucosa not near the tumour to be inspected for lesions. The detection and histological examination of tiny polypoid lesions can provide clues for diagnosis of a hereditary polyposis syndrome. On the other hand, mucinous and medullary histological types, and the presence of a heavy intra- or peritumoral lymphocytic inflammatory infiltrate, are typical features of CRCs associated with Lynch syndrome. In these cases, the IHC expression of DNA repair gene proteins (MLH1, MSH2, MSH6 and PMS2) should be examined, or the polymerase chain reaction (PCR) should be used to study MSI. Finally, a thorough search for lymph nodes buried in the pericolic adipose tissue, although a tedious task, allows correct staging of the tumour. The latest edition of the international TNM classification recommends that 12 or more lymph nodes be assessed histologically.

Table 3 Description of pre-test phase

Specimen fixation
Rapid (within 1 h of it being obtained)
Use 10 % neutral buffered formalin for <24 h:
6 to 12 h for endoscopic biopsy specimens
8 to 24 h for surgical specimens
Avoid fixatives based on alcohol (B5®, PEN-FIX®) or containing mercury (BOUIN®, ZENKER®) or microwave-based rapid fixing methods
Specimen processing
The specimen is processed following the standard protocol

Finally, in order for the results of molecular biology techniques to be considered valid, it is essential for the tumour sample to be chosen correctly, which requires involvement of the pathologist. In any case, a number of essential requirements must be borne in mind, such as: (1) it is better for tumour DNA to be extracted from the resection specimen than from the endoscopic biopsy; (2) necrotic and heavily inflamed areas should be avoided; (3) efforts should be made to manually microdissect the largest possible piece of tumour, containing as little healthy tissue as possible, to ensure that most of the DNA extracted comes from tumour cells. In the case of synchronous tumours, it must be remembered that both neoplasms should be examined as independent tumours [51].

Although there is good correlation between *KRAS* mutation results obtained with the primary tumour and metastases, it is recommended that molecular tests should also be done on metastatic tissue, as de novo mutations can arise in that tissue [52–54].

Test phase

It is currently only considered useful to know *KRAS* mutation status in order to select an anti-EGFR monoclonal antibody treatment. However, only a third of *KRAS* wild-type patients respond to treatment. Major efforts are, therefore, being made to discover new therapeutic targets offering treatment options to the non-responder patient subgroup. The signalling pathways in which most biomarkers are being studied are EGFR/Ras/Raf/Mek/Erk (MAP kinase) and PTEN/PI3K/AKT.

MSI testing

MMR pathway testing is mainly used to identify patients with Lynch syndrome. This is characterised by germ-line mutations in *MLH1*, *MSH2*, *MSH6* and, less often, *PMS2* or *EPCAM*. Also, patients with MMR-positive tumours have a better prognosis.

It is thus advisable for the MMR pathway to be tested in all patients with CRC. This should be done by first testing all patients by IHC. If expression is inconclusive, and there are defined clinical or pathological criteria highly suggestive of familial CRC, MSI testing should also be done.

IHC tests for MLH1, MSH2, MSH6 and PMS2 can identify which gene is likely to be impaired by looking at the proteins encoded. A tumour shows 'conserved expression' if nuclear staining is observed in the tumour cells; 'loss of expression' if an absence of nuclear staining is observed in the tumour cells, in the presence of a positive internal control (lymphocytes within the tumour, stromal cells, non-cancerous mucosa); and is deemed 'non-evaluable' when lack of expression is not accompanied by a positive internal control. Thus, if there is loss of MLH1 expression, other complementary markers may exist, such as the BRAF-V600E mutation or methylation of the MLH1 promoter. Almost all cases in which MLH1 promoter hypermethylation is observed are sporadic. Very rarely, germ-line methylations have also been described.

In contrast, MSI testing is done on tumour DNA. The current trend is to use a panel of five mononucleotide markers, commercially available in assay form, as this provides a more sensitive test than the Bethesda panel and does not require comparison with constitutional DNA. A tumour is deemed to exhibit instability if it has at least two altered markers.

When interpreting the results, the following points should be borne in mind:

– In 90 % of cases, loss of MLH1 and PMS2 expression is considered a sporadic event. In order to identify it as such, *BRAF* and MLH1 methylation tests can also be done.
– Loss of MSH6 expression is a secondary event, because MSH6 has a microsatellite sequence in the coding region.
– Loss of expression of MSH2 and MSH6, MSH6 alone, PMS2 alone or a tumour with instability but no loss of expression by IHC is often associated with the existence of Lynch syndrome.
– If a stable tumour displays loss of expression by IHC, the DNA extraction and IHC tests should be repeated to confirm the inconsistency.
– Patterns other than those described above are very rare and should be treated on an individual basis.

KRAS testing

The indication for *KRAS* testing may come either from the medical oncologist or from multidisciplinary groups that draw up action algorithms. When that indication exists, the pathologist should do the mutation study either when the patient has advanced disease, or even when the initial histopathological diagnosis of the tumour is being performed.

Determining *KRAS* mutation status is important because: (1) it helps to optimise the choice of candidates for specific inhibitor-based therapies, although it must be borne in mind that signalling pathways contain other factors that may also be altered, inducing cell proliferation; (2) *KRAS* mutations associated with constitutive activation confer resistance to anti-EGFR monoclonal antibody treatments; and (3) both the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) currently require *KRAS* mutation tests in CRC before anti-EGFR monoclonal antibodies are administered.

In any case, there are some situations in which *KRAS* mutation testing in CRC patients may be unacceptable,

including: (1) if there is no histological confirmation of infiltrating carcinoma by a pathologist; (2) if the sample does not reach the recommended quality requirements in terms of pre-fixing, fixing, tumour content of the sample, DNA quality, etc.; (3) if the required quality is not achieved during test and post-test phases (e.g. because of unsatisfactory internal control results, etc.); and (4) if negative results are obtained with insufficient or poor samples, i.e., below the threshold of sensitivity required by the technique used.

In order to test for *KRAS* mutation status, it is important to bear in mind the existing recommendations [55], for both the pre-test and test phases, as well as the methods used in each of them. In the test phase, for examining *KRAS* mutations and other molecular abnormalities, the first important issue is the choice of sample. In the case of *KRAS*, given the high level of agreement between mutations in the primary tumour and mutations in metastases, the most representative tumour sample can be chosen, although de novo mutations can arise in metastatic tissue [52–54]. That sample can come either from the surgical specimen, or from an endoscopic biopsy or a cytological preparation obtained by fine-needle aspiration (FNA) of metastatic lesions. One important unresolved issue is the percentage tumour content a sample must have to be tested by molecular techniques. With constant progress being made in molecular diagnostic protocols, providing ever-increasing diagnostic sensitivity, the proportion of tumour cells needed for molecular diagnosis is decreasing all the time. Nevertheless, it is recommended that the diagnosis be made on representative blocks, with at least six to ten sections of about 5 microns each. In this respect, it is advisable to select the block with the highest percentage tumour content and/or use a marker pen to indicate the areas of highest concentration of viable tumour, reflect the percentage necrosis observed in the chosen sample and then, if necessary, perform a macro-dissection of the whole sections with a histological needle and/or scalpel or a microdissection if they are minimal lesions with a very low percentage of tumour cells.

The molecular methods and techniques used to test for *KRAS* mutations are the same as for any molecular pathology study. Direct sequencing methods, such as the Sanger method, with its inherent limitations, can thus be used. This is a method of high specificity but low sensitivity. In order to use it, a greater amount of mutated tumour DNA is usually required, with a sensitivity of about 25 %. In line with direct sequencing methods, pyrosequencing is now being implemented. This requires a pyrosequencer, but has higher sensitivity, of around 5–10 % mutated DNA. However, the methods most often used are real-time quantitative PCR methods, for which various protocols and methodological approaches exist. First, the TaqMan PCR technique is an ideal method for detecting specific mutations, and has high sensitivity, at around 5–10 %. Also based on real-time quantitative PCR, the Scorpions ARMS method only detects specific mutations, with high sensitivity, even below 1 % mutated DNA.

If mutated allele enrichment techniques are needed, these methods and techniques require greater technical training and experience in the laboratories that perform them. Thus, PCR enrichment techniques exist [55], such as PNA–LNA PCR clamp, which has sensitivity of up to 0.1 % mutated DNA, or COLD-PCR, with sensitivity of even less than 0.1 %.

Finally, other methods based on mutations generating changes in restriction enzymes could be used, such as those that rely on high-performance liquid chromatography (HPLC) or high-resolution melting (HRM) analysis. Although at an early stage of development, this method is thought to offer great potential in the short to medium term.

BRAF testing

The V600E mutation is the most common change in the *BRAF* gene in CRC and can be studied simply by sequencing.

EGFR testing

For IHC tests on *EGFR* expression, various antibodies recognising different epitopes on the receptor are commercially available, and there is no unanimity or consensus about using them, which protocol the technique should follow, or how to assess the results. Perhaps for this reason, data in the literature are contradictory and it is hard to draw any conclusions. Tumour expression of EGFR as detected by IHC is heterogeneous and stronger at the deep invasion front of the tumour [56], although some authors correlate maximum staining intensity (3+) with gene amplification by means of chromogenic in situ hybridisation (CISH) techniques [57]. IHC detection of the phosphorylated or activated protein does not provide any better results than detection of EGFR expression [58].

IHC testing for EGFR is not currently used to select patients with metastatic CRC eligible for cetuximab treatment [59].

Testing for other mutations

Lack of PTEN expression by IHC techniques has been correlated with failure to respond to cetuximab [60–62]. However, other authors regard loss of *PTEN* expression as an indicator of poor prognosis [47, 63, 64]. The inconsistency of results is partly due to problems with the IHC technique, patterns of positivity (cytoplasmic vs. nuclear) and the different antibodies used [65].

As regards testing for *PI3K* mutations, according to a recent meta-analysis, mutations in exon 20, rather than

exon 9, may be a marker of resistance to anti-EGFR monoclonal antibody treatment in *KRAS* wild-type advanced CRC [66].

Finally, various antibodies for detecting p-AKT by IHC are commercially available, and there is no unanimity or consensus about how to use them [67].

Post-test phase

The reports issued must contain the necessary pathology information for TNM staging of the tumour and provide the molecular data that influence treatment. Consensus protocols drawn up by various pathologists' associations regularly review the information to be contained in pathology reports, and are updated in line with changes made to the TNM classification [68, 69]. Notable new features in the seventh edition of the TNM classification of malignant tumours are as follows [70]:

- Tumour invasion of vascular structures, whether lymphatic vessels or veins, is described under a single category of angiolymphatic invasion.
- Satellite tumour deposits are nodules or groups of cancer cells found in adipose tissue, where the regional lymph nodes draining the primary carcinoma are located.
- If the tumour deposit has a rounded margin it should be considered as a lymph node completely infiltrated by the tumour. If examination under the microscope detects remnants of the lymph node, a metastatic lymph node should also be deemed to exist. In either situation, these lesions must be added to the final calculation of metastatic nodes making up the pN score.
- However, if the tumour deposit is unevenly shaped and there is no evidence of any remnants of a lymph node, it should be regarded as satellite growth of the primary tumour or venous invasion with extravascular infiltration. In either situation, and whether one or several exist, it should be reported as a tumour deposit in category pN1c in those carcinomas without metastatic lymph nodes with pT1 or pT2.
- The presence of tumour in the peritoneum should be regarded as metastasis (M1b).

It is recommended that the report should include the results of IHC tests for protein expression from DNA repair genes (MLH1, MSH2, MSH6 and PMS2), molecular markers of MSI and *KRAS* and *BRAF* gene mutations [68].

Internal and external quality controls

It is advisable for laboratories conducting either IHC or molecular biomarker tests to take part in quality control programs, such as the one set up by SEAP.

It should be up to the health authority in each self-governing region to set the requirements for laboratory accreditation and certification. If it is agreed that these techniques should be carried out at certain referral centres according to their level of accreditation and certification, enough time should be allowed to enable those sites interested in doing so to achieve the requisite accreditation and certification.

Common issues

Recommended and acceptable timescales

How quickly the results are delivered depends on: (1) the time taken to obtain the sample after it has been requested; (2) how long it takes for the sample to be sent to the laboratory conducting the tests; (3) the time taken for the result to be obtained and issued once the laboratory has received the sample; and (4) how soon the result sent out is received by the doctor who ordered it. Given the complexity of the procedures and the involvement of various different health professionals, a work flow must be established at each site to optimise the test process.

Considering the importance of *KRAS* mutation status to decision-making in patients with metastatic CRC, the total time taken to obtain this test result should be about seven working days.

Legal implications

Handling genetic information has major repercussions from the therapeutic and prognostic point of view. However, it also has legal and ethical implications, which must be understood. The aim of these is for patients' confidentiality and autonomy to be respected.

In this respect, among other applicable laws, the Biomedical Research Act of 3 July 2007 and the Royal Decree 1716/2011 released on December 2011, and that will be effective on June 2012, deals with how to handle this type of information [71]. This law addresses some highly practical issues worth noting in this consensus statement, especially in such a sensitive area as hereditary cancer. As mentioned above, some molecular analyses, such as MSI tests or protein expression from some repair genes, can help to identify which CRC patients have a hereditary pattern.

From a legal point of view, patients due to undergo this type of test should be told that a positive result might mean an increased risk of developing not just CRC but some other tumour associated with Lynch syndrome, such as endometrial cancer. On the other hand, this genetic information affects other members of the family, so they may get involved in the process. For all these reasons, patients must sign an informed

consent giving permission for germ-line genetic tests to be done. They must also be offered the opportunity of appropriate genetic counselling depending on the result.

Points covered in the informed consent for this sort of study must include: (1) the aim of the study; (2) where it is to be done and what will happen to the biological sample at the end of it; (3) who will have access to the result; (4) a warning about the possibility of unexpected findings; (5) potential implications for family members; and (6) an undertaking to provide genetic counselling. If the ideal patient for tests of this type has already died, they can still be done, providing this is not expressly forbidden and might potentially benefit his or her biological relatives (with their prior permission, obviously).

It must be remembered that, even though the patient gave informed consent in advance, it can be withdrawn whenever the subject so wishes. However, when this information is necessary to prevent serious harm to the health of biological relatives, the affected individuals or the legally authorised representative can be informed.

Test results should be kept for at least 5 years. In the absence of any request from the person concerned, data should be kept for as long as necessary to preserve the health of the subject or related third parties.

Finally, whenever a genetic study is done for health purposes, the person concerned must be guaranteed appropriate genetic counselling. This should address issues concerning the risk of developing cancer, for both the person concerned and his or her offspring, as well as ways of controlling that risk, reproductive matters and psychological management.

Conclusions

CRC is an ideal model for studying the molecular pathogenesis of cancer, because of the ease of obtaining a tumour biopsy and the specific genetic changes that occur at each stage of its development. This has led to the identification of prognostic and predictive biomarkers that help with the clinical management of this condition, in both the diagnostic and the therapeutic phase.

The need for appropriate biomarker testing at each clinical phase of CRC (genetic counselling, localised CRC subjected to radical surgery and metastatic CRC) led to the production of these guidelines, which review the level of evidence for tests on each of these biomarkers. Other important issues addressed in this consensus statement are evaluation of the organisational requirements and quality controls needed for testing these biomarkers, which particularly affect the pathology department, so that a rigorous diagnosis can be reached as quickly as possible, as well as the legal implications of testing certain biomarkers.

As regards genetic counselling, most colorectal cancers are caused by inactivation of the *APC* gene, by either sporadic or germ-line mutation, and involve no MSI. For this reason, patients at high risk of developing CRC can be identified by doing genetic tests to detect specific germ-line mutations.

In localised CRC, MSI is a marker of susceptibility to hereditary CRC, but it is also a strong predictive factor for deciding on adjuvant treatment in patients with stage II disease. It is well established that its presence confers a better prognosis. However, predictive value for 5-FU resistance is more controversial. On the other hand, although the ColoPrint® and Oncotype Dx® gene expression signatures have been shown to have prognostic value, no consensus yet exists concerning their use in clinical practice.

As regards metastatic CRC, testing for mutation status of the *KRAS* oncogene, which is present in 40 % of patients with CRC, is obligatory before administering anti-EGFR treatment, such as cetuximab and panitumumab, given its negative predictive value for response.

The recommendations laid down in this consensus statement are confined to clinical healthcare practice, although other biomarkers that may shortly enter the clinical routine, if their predictive value is confirmed, are also evaluated, such as mutations in *BRAF*, *EGFR*, *PI3K*, *PTEN* and *AKT*. This document is the result of oncologists and pathologists working together in a multidisciplinary fashion to achieve a consensus on the rational use of biomarkers in CRC, and methods for testing them. It lays the foundation for major developments and increasing complexity envisaged in the future, which may lead to individually tailored treatment for the patient, with more effective therapies.

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