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Biomarker in CRC

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Abstract

In the last 20 years improvements in metastatic colorectal cancer treatment lead to a radical raise of outcomes with median survival reaching now more than 30 months. Despite that, the identification of predictive and/or prognostic biomarker still represent a challenging issue and up today, although clinician and researchers might face with a deeper knowledge of biological mechanisms related to colorectal cancer, many evidences underline the heterogeneity and the dynamism of such disease. In the present review we describe the road leading to the discovery of *RAS* mutations, *BRAF* V600E mutation and microsatellite instability role in colorectal cancer; secondly we discuss some of the possible major pitfalls of biomarker research and lastly we give new suggestions for future research in this field.

Keywords

colorectal; biomarkers; RAF; BRAF; MSI; clinical trials; liquid biopsies

Introduction

Colorectal cancer (CRC) is the second highest cause of cancer mortality both in the United States and in Europe. About 25% of cases present with metastases at the time of diagnosis, while another 25% of patients will develop metastases during the course of the disease [1,2].

The median overall survival (OS) of metastatic colorectal cancer (mCRC) patients in the last 20 years has remarkably improved from about 6-12 months [3] to over 30 months in the last presented first-line clinical trials [4-7]. Such results derive from the development of new intensive and/or tailored therapies, which incorporate cytotoxic drugs and targeted therapies such as the anti-epidermal growth factor receptor (EGFR)- monoclonal antibodies (MoABs), cetuximab and panitumumab, and the anti-angiogenic bevacizumab [8-10] the integration of medical treatments with more and more effective locoregional and surgical approaches [11] and from a deeper knowledge of CRC biology [12]. Moreover, steps forward have been done thank to the approval of new drugs, such as aflibercept [13], regorafenib [14] TAS 102 [15] and ramucirumab[16].

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Despite those improvements, the identification of the most effective treatment for an individual patient is still mainly based on clinical considerations such as symptoms, performance status, extent of disease, patients' preferences and treatment history, while the identification of predictive and/or prognostic biomarkers able to guide treatment decision stands as a challenging issue in the management of CRC patients. According to the NIH biomarker definition working group a biological marker (biomarker) is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. [17] A prognostic marker influences patients' outcome regardless of treatment, whereas a predictive biomarker identifies patients more likely to benefit from a specific treatment. Personalizing treatment according to biomarkers might have important clinical utility and public health significance, leading to improved therapeutic outcome through a reduction of harm and/or costs associated with treatments.

In the first part of the review we will summarize the role and the discovery history of the *RAS*, *BRAF* and microsatellite instability (MSI), that represent at the moment the only validated biomarker in CRC. In the second part we will focus on possible reasons of biomarker discovery failure and we will propose our way-out strategies to make translational research in this field more effective.

RAS: The never ending story

Cetuximab and panitumumab received the FDA approval in 2004 and 2006 respectively after the demonstration of efficacy in randomized trials for mCRC patients in advanced lines of treatments. In comparison with best supportive care (BSC), cetuximab was associated with a significant improvement in OS (hazard ratio (HR)=0.77; 95% confidence interval (CI) 0.64-0.92; P=0.005) and in progression-free survival (PFS) (HR=0.68; 95% CI 0.57-0.80; P<0.001)[18]; similarly, panitumumab demonstrated an improvement of PFS compared to BSC (HR=0.54; 95% CI 0.44-0.66; P< 0.0001). No difference was observed in terms of OS (HR=1.00; 95% CI0.82-1.22), but 76% of BSC patients received panitumumab in the cross-over study[19]. Interestingly, looking at PFS curves, a clear subgroup effect was hypothesized: it seemed that more than 50% of patients in the experimental arm did not derive any advantage from either cetuximab or panitumumab. Moving from such consideration the search for predictive markers of response or resistance to anti-EGFRs started.

EGFR expression tested by immunohistochemistry (IHC) on surgical specimens was the first proposed predictive factor of response to anti-EGFRs [20,21]. However some authors described clinical responses to cetuximab in EGFR-negative tumors [22,23]. Technical issues such as tissue fixation, storage time [24], non-homogeneous pattern of expression, personal interpretation of results and biological limitations such as lack of concordance between primaries and related metastases [25] have been proposed as possible limitations of the adoption of EGFR expression measured by means of IHC as a predictive factor of anti-EGFRs activity. Similarly, due to reproducibility issues and high costs, EGFR amplification detected by FISH was not deemed as a reliable biomarker [26-29].

The revolution came from the discovery of *KRAS* exon 2 (codon 12 and 13) mutations as negative predictive factor of response to anti-EGFRs. *KRAS* is a GTPase protein involved in

signal transduction, cellular growth, differentiation, proliferation and survival and is encoded by RAS family member genes [30,31]. Mutations of *RAS* lead to a defective GTPase activity that causes a hyperactivation of the signalling cascade [32] determining an uncontrolled activity of the downstream effectors, such as RAF proteins and MAP-kinases. *KRAS* mutations are found in about 40-50% of mCRC patients and more frequently affect exon 2 [33,34].

First evidence of the role of *KRAS* exon 2 mutation were obtained in small retrospective series [29] and were confirmed in post hoc analyses of phase III randomized trial, both in advanced setting [18,35,36] and in first line [37,38] and subsequently in meta-analyses[39,40]. In 2008 FDA and EMA restricted the use of anti-EGFRs to *KRAS* exon 2 wild-type patients; however, it seemed that only a small percentage of wild-type patients derived benefit from those drugs and researchers pursued the effort of refining patients selection to anti-EGFRs.

Additional rare *RAS* activating mutations, involving exon 3 (codon 59 and 61) and 4 (codon 117 and 146) of *KRAS* and exon 2 (codon 12 and 13), 3 (codon 59 and 61) and 4 of *NRAS* (codon 117 and 146) were deemed as possible additional negative predictive factors. Preliminary evidences came from a retrospective series of 87 *KRAS* exon 2 wild-type mCRC patients treated with irinotecan and cetuximab. *KRAS* codons 61 and 146 mutant patients showed a lower response rate (0 versus 37%, P 0.047) and worse PFS (HR= 0.45, P=0.023) compared to wild-type patients [41]. Moreover, in a large dataset of chemo-refractory mCRC patients (N=733) treated with cetuximab and chemotherapy in seven European countries, it was shown that among *KRAS* wild-type patients, carriers of *NRAS* mutations had a significantly lower response rate than *NRAS* wild-type (7.7% vs 38·1% respectively; OR=0.14, p=0.013) [42]. Supplementary confirmatory data were subsequently presented in a large (N=1,630) phase III randomized study of first-line chemotherapy plus or minus cetuximab [43]. The definitive turning point was the publication of the extended *RAS* analyses data from the PRIME trial comparing FOLFOX plus or minus panitumumab in first-line mCRC patients. A detrimental effect of the addition of panitumumab to chemotherapy was observed in patients carrying *RAS* mutations (HR for PFS=1.31 p=0.008, p for interaction <0.002; HR for OS=1.21 p=0.04, p for interaction=0.001). Moving from such results the use of anti-EFGR was restricted to RAS wild-type patients by regulatory agencies.

After that, the role of the extended *RAS* mutations' status was evaluated in all recent randomized trials (main results are summarized in Table 1)[44-46] and was also confirmed in a meta-analyses: no PFS or OS benefit was evident with use of anti-EGFR for tumors carrying any *RAS* mutation (p>0.05) and results were consistent between different anti-EGFR agents, lines of therapy and chemotherapy partners [47].

In terms of clinical outcome, the effect of *RAS* mutations is still a matter of debate. In the metastatic setting the prognostic effect of such mutations might be underestimated due to the well-known predictive effect [43,48]. In the adjuvant setting a negative prognostic effect has been hypothesized [49,50] and subsequently confirmed in a large pooled analysis from the PETACC8 and N0147 trials, where *KRAS* exon 2 mutations were identified as independent predictors of shorter time to recurrence (HR=1.60, 95% CI 1.60-1.83, p<0.0001) and OS

(HR=1.52, 95% CI 1.29-1.79, p<0.0001) among patients with stage III microsatellite stable (MSS) tumors [51]. A recent meta-analysis investigated the impact of *KRAS* mutation on outcomes in patients undergoing liver resection. *KRAS* mutation was negatively associated with OS (HR=2.24, 95% CI 1.76-2.85) and relapse free survival (HR=1.89, 95% CI 1.54-2.32) in this setting, where the effect of *KRAS* mutation might be independent to the use of anti-EGFRs [52].

According to all the previous observations, we should acknowledge that *RAS* evaluation methods become of paramount importance in the management of CRC patients. Genetic tests should be conducted in high qualified and certified laboratories and as soon as possible after diagnosis of CRC. The American Society of Clinical Oncology recently provided a Provisional Clinical Opinion Update regarding *RAS* mutational testing of CRC tissue and is developing a new guideline for marker testing in collaboration with the College of American Pathologists, the Association for Molecular Pathology, and the American Society for Clinical Pathology [53]. Among different detection methods, sanger sequencing is deemed as a standard method for *RAS* testing but requires at least 10-25% of *RAS* mutant neoplastic cells in the sample for reliable detection [54]; pyrosequencing has a sensitivity <5% but sequencing error rate may be around 4 to 25% [55]. Next generation sequencing platforms (I.E. MiSeq - Illumina Sequencing Systems; Ion Torrent - Life Technologies) allow for the identification of single-nucleotide changes, insertions, deletions, and even translocations of multiple genes and loci in a single PCR reaction; sequencing error rates are around 1% and usually 1-5% of *RAS* mutant neoplastic cells are needed. Those methodologies are expected to completely replace older methods in the next few years[56].

Detection of *RAS* mutations through liquid biopsies is the striking news of the last years. Circulating free DNA can be extracted from blood and might allow for detection of tumor-specific genetic aberrations, leading to assessment of the presence of residual disease, recurrence and primary/acquired resistance [57]. This method allows catching the dynamisms and the heterogeneity of tumors and identifying emerging *RAS* mutations leading to acquired resistance to anti-EGFRs. *KRAS* wild-type patients treated with an anti-EGFR develop mutated *KRAS* clones in blood during EGFR blockade. Interestingly, such mutated clones decline after withdrawal of treatment, leading to a possible regain of drug sensitivity [58]. Droplet digital PCR and beaming techniques represent new tools able to identify such mutations[59-62]. These approaches have been applied in research, but at the moment they are not applicable in routinely clinical practice for *RAS* testing since their clinical validity is still under evaluation.

BRAF history

BRAF is an important player in the EGFR-mediated downstream signalling pathway. It is activated by *RAS* and is able to affect cell growth, proliferation, and differentiation through the activation of MAP kinase pathway, but also able to influence apoptosis (through the regulation of BCL-2), cell migration (through RHO small GTPases) and survival (through the regulation of other pathways such as BCL-2, RHO and HIPPO) [63]. *BRAF* V600E mutation affects 8 to 10% of mCRC patients. Interestingly, a higher rate of *BRAF* mutation incidence (21%) was reported in a cohort of prospectively collected unselected mCRC

patients with a high rate of poor performance status (39% with PS 2-4) and advanced age (37% with age>75), thus underlining that incidence *of BRAF* mutant patients might be underestimated in clinical trials [64].

The story of *BRAF* mutation started in 2008 when Di Nicolantonio et al suggested a possible role as markers of resistance to anti-EGFR in a small retrospective series [65]. Subsequently *BRAF* gained a prominent role as a negative prognostic factor in the metastatic setting where *BRAF* mutant patients showed a median OS lower than 12 months in multiple series [66-69]. Specific clinical features such as advanced age, female gender, right sided-primary location, mucinous histology, microsatellite instability, a high rate of nodal and peritoneal metastases [67,70-72] a peculiar gene signature [73] and a specific carcinogenesis pathway [74] have been also associated with *BRAF* V600E mutation. Moreover, when liver metastases are radically resected, *BRAF* mutated tumors often relapse rapidly, due to the occurrence of extrahepatic lesions [75,76].

In the adjuvant setting the prognostic role of *BRAF* mutation is still controversial, due to the strong association with MSI. In the previously reported pooled analyses of PETACC8 and N0147 trials, *BRAF* mutation emerged as a strong independent negative prognostic factor for time to recurrence (HR=1.49, 95% CI 1.19-1.87, p=0.0005) and OS (HR=1.72, 95% CI 1.33-2.22, p<0.0001) among stage III CRC microsatellite stable patients. [51]

Recently, the impact of rare mutations of *BRAF* (codons 594 and 596), occurring in <1% of CRCs, was investigated. Such mutations were associated with rectal primary tumor location, non-mucinous histology, microsatellite stability and lack of peritoneal spread. Moreover no negative prognostic impact was observed (*BRAF* 594 or 596 mutant vs *BRAF* V600E median OS 62.0 versus 12.6 months; HR=0.36, 95% CI 0.20-0.64, p=0.002). [77]

The debate is still open about the predictive effect of lack of response to anti-EGFRs. Confirmatory findings come from the large dataset of chemo-refractory mCRC patients (N=733) included in the European Consortium analyses: response rate to cetuximab plus or minus chemotherapy was 8.3% (2/24) versus 38.0%(124/326) in *BRAF* mutant vs wild-type respectively (OR=0.15, 95% CI 0.02-0.51; p=0.0012) [42]. Conversely, in non-prespecified retrospective analyses of several phase III trials, *BRAF* V600E mutation failed to provide evidence for utility as predictive biomarkers due to the small number of patients and lack of statistical power [78,79]. Main results are summarized in table 2. Recently [80,81] in a meta-analysis of clinical trials evaluating the efficacy of anti-EGFRs, Rowland et al showed that among patients carrying *RAS* wild-type-*BRAF* mutant tumors the HR for OS benefit was 0.97 (95% CI; 0.67–1.41) versus 0.81 (95% CI; 0.70–0.95) for RAS wild-type-BRAF wild-type with a negative P for interaction (p= 0.43). In terms of PFS the HR was 0.86 (95% CI; 0.61–1.21) and 0.62 (95% CI; 0.50–0.77) in the 2 groups respectively, with a positive interaction (p= 0.07) [78]. On the other side, in a meta-analysis including 9 phase III trials and one phase II trial (6 first-line, 2 second-line trials and 2 trials involving chemo refractory patients), Pietrantonio et al demonstrated that the addition of anti-EGFR in *BRAF* mutant patients did not significantly improve PFS (HR=0.88; 95CI 0.67-1.14, p = 0.33), OS (HR=0.91; 95% CI 0.62-1.34, p= 0.63) and ORR (relative risk=1.31; 95% CI 0.83-2.08, p= 0.25) compared to control regimens.

In our opinion those results show that anti-EGFRs do not demonstrate a clear outcome benefit in *BRAF* mutant patients; moreover those drugs might affect quality of life in terms of toxicity and treatment costs in this group of patients. Such evidences underline the importance of the identification of tailored treatment options. In the first line setting FOLFOXIRI plus bevacizumab represents the most promising treatment option in clinically selected *BRAF* mutant patients[4,82]. Many trials have been conducted in the last few years aiming at the identification of possible effective *BRAF* inhibitors for mCRC patients. Unfortunately, results are not as exciting as those observed in other diseases such as melanoma; more recently a huge effort has been made in order to identify more promising combinations of drugs [83-87]. Other articles in this issue focus on *BRAF* directed treatment.

MSI as prognostic, predictive and therapeutic target

DNA MSI phenotype is caused by deficient DNA mismatch repair (MMR) as a consequence of germline mutations in MMR genes or, more commonly, epigenetic silencing of the MLH1 gene with frequent mutations in the *BRAF* oncogene. During DNA synthesis, MMR proteins repair base pair mismatch errors in tandemly-repeated sequences named microsatellites, in order to maintain genomic stability. Deficient MMR results in the production of truncated, nonfunctional protein or loss of a protein which causes MSI phenotype

Germline mutations in the MMR genes (MLH1, MSH2, MSH6, and PMS2) lead to an autosomal dominant hereditary syndrome named hereditary nonpolyposis colorectal cancer (HNPCC), or Lynch syndrome [88,89]. Patients with Lynch syndrome are more likely to present synchronous tumors and at younger ages (between 20 and 30 years) compared to other CRC patients, and rarely carry *BRAF* mutation[90]. Moreover they show higher risk to develop not only CRC but also endometrial, gastric, ovarian, urinary tract and small intestine cancers [91].

The MSI phenotype is present in about 15% of colorectal cancers (CRC) – its frequency is higher in stage II (about 20%) than in stage III (about 12%) and decreases in the metastatic setting (4%) [92]. MSI tumors share specific clinical and pathologic features, such as right-sided primary location, poor differentiation, mucinous histology, and increased numbers of tumor-infiltrating lymphocytes [93].

MSI testing can be performed by means of immunohistochemistry (IHC) or PCR-based assay. A panel of microsatellite markers have been validated and recommended as a reference panel for PCR analyses[94].

In terms of prognosis MSI status has been shown to confer a good outcome in patients with localized disease. Those data derive from retrospective analyses of adjuvant treatment clinical trials or clinical trials population-based studies and meta-analyses [88,95-97]. Subgroup analyses of the QUASAR trial, evaluating the efficacy of 5-FU adjuvant treatment compared to observation in the adjuvant setting, showed that recurrence rate in patients with MSI tumors was 11% (25/218), compared to 26% (438/1695) in the MSS cohort [risk ratio (RR), 0.53; 95% CI, 0.40-0.70] [98]. A large meta-analysis of pooled data from 31 trials, including 1972 MSI out of 12782 stage I-IV CRC patients, showed an association between MSI and favourable prognosis both in terms of OS (OR=0.6, 95% CI 0.53-0.69, p<0.0001) and

disease free survival (OR=0.58, 95% CI 0.47-0.72, p<0.0001) [99]. In the metastatic setting a recent large pooled analysis of four randomised first-line phase III trials (CAIRO, CAIRO2, FOCUS and COIN) evaluated the impact of MSI and *BRAF* in terms of outcome. PFS and OS were significantly reduced in the MSI cohort, in comparison to the MSS cohort (median PFS 6.2 vs 7.6 months respectively; HR=1.33, 95% CI 1.12-1.57, p=0.001; median OS 13.6 vs 16.8 months respectively; HR=1.35, 95% CI 1.13-1.61; p=0.001). The negative prognostic effect of the *BRAF* mutation was confirmed; no interaction between the two markers was demonstrated, thus suggesting that the poor prognostic value of MSI is driven by the *BRAF* status in this setting [100]. All the available evidences suggest that the prognostic impact of MSI could be stronger in earlier stage than in advanced tumors [101,102].

More controversial is the role of MSI as predictive factor of response to 5-FU-based therapy [95,96,98,103-106]. The most accepted interpretation of the role of MSI in the adjuvant setting derives from results of the ACCENT database. Such analyses were aimed at the evaluation of the impact of MSI in stage II and III CRC among 17 adjuvant trials comparing surgery vs surgery followed by 5-FU-based therapy. Among patients treated with surgery alone those with stage II and MSI tumors showed better time to recurrence (TTR) (5 years TTR 89 vs 74%; HR=0.35, 95%CI 0.15-0.80, p=0.013) and OS (5 years OS 90 vs 78%; HR=0.37, 95%CI 0.17-0.81, p=0.013) compared to those with MSS; similar results were observed when comparing MSI vs MSS stage III patients (5 years TTR 60 vs 47%; HR=0.79, 95%CI 0.45-1.39, p=0.41; 5 years OS 59 vs 54%; HR=0.84, 95%CI 0.49-1.43, p=0.51). Moreover in stage III CRC patients, a significant survival benefit for 5-FU monotherapy vs. surgery alone was seen in patients with MSS tumors (5-year TTR =64% vs. 47%), but also in patients with MSI tumors (5-year TTR =72% vs. 60%). The authors suggest that chemotherapy is not recommended for patients with stage II and MSI tumors due to their excellent prognosis, while stage III patients should receive adjuvant treatment irrespective of MSI status [107].

Recently MSI gained a prominent role in the metastatic setting due to the discovery of immunotherapy susceptibility. A phase 2 study evaluated the clinical activity of pembrolizumab, an anti–programmed death 1 immune checkpoint inhibitor, in 41 patients mCRC with MSI and MSS. Response rate and PFS rate were 40% and 78% respectively among MSI cases while 0% and 11% among MSS cases. Advantages in terms of PFS (HR=0.10, p<0.001) and OS (HR=0.22, p=0.05) were also observed in MSI compared to MSS patients[108]. At the moment the mechanisms explaining such results are not fully understood; the most validated hypothesis suggests a role for the high mutation rate observed in MSI tumors. Those mutations lead to a high tumor neoantigens rate and higher lymphocyte infiltrate, and finally reveal a role of patient's immune system[109,110]. Detailed explanations of such mechanisms will be provided elsewhere in this issue.

Why is research of biomarkers so hard?

Except for the above-mentioned *RAS*, *BRAF* and MSI, at the moment there are no other relevant biomarkers in CRC.

Biomarker validation represents a major issue in translational oncology. Many attempts have been made in order to find adequate biomarkers for antiangiogenic drugs with disappointing

results. Among them many pharmacogenetic studies have been conducted aiming at the evaluation of *VEGFA* SNPs as predictors of bevacizumab's efficacy [111-113]. Our group carried out a large, retrospective genotyping analysis of 4 SNP in VEGFA gene –2578 C > A, −1498 C > T (rs833061), 936 C > T, 405 C > G for 218 mCRC patients, 111 treated with FOLFIRI plus bevacizumab and 107 treated with FOLFIRI alone. In the bevacizumab group, patients carrying the VEGF rs833061 T/T genotype showed shorter PFS (HR=2.13, (95 % CI 1.41–5.10), p=0.0027), while none of the SNPs was associated with outcome in the control group. Due to the positive interaction between VEGFA rs833061 and treatment effect, a possible predictive role for the SNP was suggested [114]. In order to confirm this data we subsequently conducted a prospective validation study and 424 patients treated with first-line FOLFIRI plus bevacizumab were enrolled. At the univariate analysis no differences in PFS according to VEGF rs833061 C/T variants were observed (p=0.38), so the primary end point was unmet and the predictive effect of VEGF rs833061 C/T SNP was denied. There might be many possible reasons for these negative results. The hypothesis of identifying a single *VEGFA* SNP as a strong predictor of bevacizumab's efficacy was maybe too naïve, since more and more evidences underline the importance of tumor heterogeneity, the complexity of bevacizumab mechanism of action and of the assessment of anti-angiogenic treatments response [115-117]. Disappointing results were also derived from the research of predictive factors for anti-EGFRs efficacy. Despite many attempts, the predictive effects of *PIK3CA* mutations and of PTEN loss are still unclear in mCRC receiving anti-EGFRs due to the lack of validation studies.

As exemplified above for *RAS*, *BRAF* and *MSI*, prognostic biomarkers might emerge from small retrospective studies, but deserves robust multi-site validation. Predictive biomarkers require even more extensive data for validation derived from large randomized clinical trials and meta-analysis. Clinical trials are often not adequately powered for biomarker discovery and validation; moreover statistical correction is needed for provisional data to avoid the risk of false-positive associations, and every effort should be made to have adequate validation sets. From a clinical and methodological perspective, researchers should do their best to avoid false positive results and look at their data in a rigorous and critical way because their preliminary results would lay the basis for the next step of confirmatory validating trials. [118-120].

Besides statistics, technical issues puzzle biomarker research. In particular when adopting new technologies such as next generation sequencing, there is no consensus on sample requirements and the debate is open on whether archived tumors, fresh tissue biopsy or a liquid biopsy should be selected for profiling. Moreover assays adopted to identify biomarkers require rigorous standardization and quality assurance evaluation performed in certified laboratories [121].

With the emergence of more sophisticated gene expression techniques, several groups tried to develop potential molecular classifications of CRC able to simplify biomarker research and disease stratification. Recently the CRC Subtyping Consortium re-analysed existing gene expression-based CRC subtyping algorithms and developed a new molecular classification by cross-comparing different subtypes and resolving inconsistencies in number and interpretation of previous series. Key biological features, such as mutation, copy

number, methylation, microRNA and proteomics as well as correlation with outcome were described. Four colorectal molecular subgroups (CMS) were identified: CMS1 (14% of CRC) associated with MSI, hypermutated phenotype, *BRAF* mutation, immune activation and worse survival after relapse; CMS2 (37%) with epithelial signature, high number of somatic copy number alterations, marked WNT and MYC signaling activation; CMS3 (13%) with epithelial signature, evident metabolic dysregulation and *KRAS* mutations; and CMS4 (23%) with mesenchymal pattern, prominent transforming growth factor–beta activation, stromal invasion and angiogenesis and worse relapse-free and overall survival[12]. This new classification represents a step forward to a better understanding of CRC biology and a deeper knowledge will be developed in the next feature thanks to the integration with 'omics' data and new treatments. A potential weakness of this classification is the inclusion of stage I to IV CRC patients, thus showing a too simplified and static picture of CRC.

As a matter of a fact, tumor heterogeneity is another significant concern in the biomarker research field. More and more evidences describe genetic and epigenetic differences between tumors of the same type in different patients, between cancer cells within a tumor and in the same tumor during disease progression. Such modifications might lead to different treatments' responses and possible mechanisms of acquired resistance to therapies. In this panorama liquid biopsies and dynamic biomarkers might be a valuable tool able to catch tumor heterogeneity and dynamism across subsequent lines of treatment.[115,122,123]

Possible ways out

The increasing complexity of CRC biology knowledge, the more advanced sequencing techniques and the emergence of new drugs targeting genetic aberration lead researchers to develop new tools to able to increase the quality of biospecimens collection and the integration of biomarkers in early phases clinical trials.

In the past few years many efforts have been made in order to improve tissue collection. In clinical trials the collection of bio- specimens or their derivatives (such as extracted RNA, DNA, or proteins) is becoming mandatory since it can help identify molecular markers of sensitivity or resistance to new drugs. Moreover, the concept of biobanking specimens emerged as new tool. It refers to the collection of clinical biopsy-type specimens (liquid biopsies or biopsies of recurrent primary or metastatic tumours) collected from patients at distinct time points and in pre-specified time points of cancer progression and treatment, which are subsequently made ready for analysis through new high-throughput technologies. Large-scale international initiatives, such as the International Cancer Genome Consortium, tried to standardize the collection and storage of biospecimens to limit pre-analytical variability and to ensure sufficient quality for analysis of samples [124]. Recently a platform named SPECTAcolor (Screening Patients for Efficient Clinical Trial Access in advanced colorectal cancer) has been built up among European country with the aim of prospectively biobanking fully annotated tumour samples and analysing possible biomarkers from patients with mCRC and finally develop personalized treatments based on the results of the tissue sampling analysis. This approach allows the improvement of sample collection, data analyses and biomarker research quality and the enrolment of patient from all over Europe to new molecularly driven trials with a huge economic convenience.

Facing with a so big amount of genomics data, many efforts have been made in order to develop more efficient and effective cancer drugs development strategies. Clinical trials with different design are used in very early drug development phases. Patient selection according to tumor genomic profile may be adopted in dose escalation and/or cohort expansion stages of phase I trials when biomarker have strong scientific background and preclinical data.

Umbrella trials involve the testing of different drugs targeting different mutations either in a single cancer subtype or in a variety of cancer subtypes. These studies typically utilize an individualized treatment plan formulated after analysis of the molecular profile of each patient's tumor. A prespecified series of treatment is used with a refined molecularly guided decision tree or algorithm. Among umbrella trials, the Cancer Research UK has developed the FOCUS 4; in this study, mCRC patients after the first 16 weeks of first-line chemotherapy are randomized to receive treatment according to results of biomarker panel assessment or placebo. Primary objectives are PFS and OS[125].

Basket trials are genotype-focused designs where a single drug on a specific mutation or mutations is tested in different cancer types. Each cohort is analyzed separately but in the same clinical trial. If the treatment under study demonstrates a signal of efficacy in a particular cohort, the cohort can be expanded to enrol more patients with the identified cancer type, while cohorts that do not demonstrate efficacy can be closed. This approach is advantageous when the mutation is rare because it allows flexibility for a combination of multiple, independent, small studies within a single trial. The intent of basket trials can be either exploratory or for registration. Among basket trials, Novartis developed the SIGNATURE study, the enrolment started in 2013 and preliminary results were presented at last ASCO Congress. Primary objective of the study is clinical benefit (SD or better for ≥ 16 weeks). Patients receive a specific drug according to the molecular profile. The most common tumor types involved are CRC and non–small cell lung cancer. The most frequent genetic alterations among treated patients were *RAS* mutation (68%), *PIK3CA* mutation (55%), and PTEN loss (41%). Preliminary activity was observed in various tumors [126].

“Hybrid” trials represent a mix of umbrella and basket trial design frameworks, a single protocol might include either multiple umbrella subtrials (same histology, different molecular aberrations), or multiple basket subtrials (same molecular aberrations, different histologies). The NCI developed 2 examples of such trial, the NCI-MPACT (“Molecular Profiling-Based Assignment of Cancer Therapy”) and NCI-MATCH (Molecular Analysis for Therapy Choice). The first one identifies genetic alteration and randomizes patients to receive a treatment that targets that alteration or a different non target cancer treatment. Primary endpoints are overall response rate (ORR) and PFS. Current targeted drugs studied in MPACT include ABT-888 (PARP inhibitor) with temozolomid; AZD1775 (MK-1775) (Wee1 inhibitor) with carboplatin and trametinib (MEK inhibitor)[127]. The second one analyzes patients’ tumors to determine whether they contain actionable mutations and assigns treatment based on the abnormality. It's a phase II and primary endpoint is ORR, enrollment started in August 2015, target accrual is 3000 patients[128].

CONCLUSION

The deeper understanding of CRC biology and the large amount of data obtained from molecular profiling and 'omics' techniques point out colon cancer as a heterogeneous disease with no driver mutations.

Translational research is focusing on the early identification of biomarkers during clinical development of new anticancer drugs. Such an approach should inform the design and conduct of early-phase clinical trials and allow consistency between populations in early-phase and phase III clinical trials.

Providing a benchmark to the international community based on a set of common principles for the integration of biomarkers into trials is important to facilitate exchange of data, promote quality, and accelerate research while respecting local approaches and legislation. Recently a risk-management approach was developed by an NCI, NCRI, and EORTC working group in order to effectively integrate biomarkers in clinical trials. [129]

Finally, it is important to understand that molecular profiles are dynamic and might change under treatment pressure, thanks to the development of liquid biopsies we are now able to molecularly monitor our patients in the real time and to identify molecular mechanism of escape informing us of possible novel treatment options. [130].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Results of main phase III trial according to RAS mutational status

Study & Setting	Therapy		RAS status	N pts		mPFS				mOS				ORR		
	Exp	Ctr		Exp	Ctr	Exp (mos)	Ctr (mos)	HR	p	Exp (mos)	Ctr (mos)	HR	p	Exp (%)	Ctr (%)	p
CRYSTAL*[1] *(I Line)	FOLFIRI+C	FOLFIRI	wt	178	189	11.4	8.4	0.56	0.0002	28.4	20.2	0.69	0.0024	66	39	<0.0001
			mut	246	214	7.4	7.5	1.10	0.47	16.4	17.7	1.05	0.64	31.7	36	0.40
PRIME*[2] *(I Line)	FOLFOX+P	FOLFOX	wt	259	253	10.1	7.9	0.72	0.004	25.8	20.2	0.77	0.009	NA	NA	NA
			mut	272	276	7.3	8.7	1.31	0.008	15.5	18.7	1.21	0.04	NA	NA	NA
FIRE-3*[3] *(I Line)	FOLFIRI+C	FOLFIRI+B	wt	171	171	10.4	10.2	0.93	0.54	33.1	25.6	0.70	0.011	66	60	0.32
			mut	NA	NA	7.5	10.1	1.31	0.085	20.3	20.6	1.09	0.60	38	51.2	0.097
CALGB/SWOG 80405*[4] *(I Line)	FOLFOX or FOLFIRI+C	FOLFOX or FOLFIRI+B	wt	270	256	11.4	11.3	1.10	0.31	32.0	31.2	0.90	0.40	69‡	54‡	<0.01
			KRAS wt exon 2 / all RAS mt	53	42	NA	NA	NA	NA	28.7	22.3	0.74	0.21	NA	NA	NA
20050181*[5] *(II line)	FOLFIRI+P	FOLFIRI	wt	204	211	6.4	4.6	0.70	0.007	16.2	13.9	0.81	0.08	41	10	NA
			mut	299	294	4.8	4.0	0.86	0.14	11.8	11.1	0.91	0.34	15	13	NA

Exp: experimental arm; Ctr: control arm; N pts: number of patients; B: bevacizumab; C: cetuximab; P: panitumumab; HR: hazard ratio; mos: months; NA: not applicable; mPFS: median progression free survival; mOS: median overall survival; ORR: Overall response rate

Table 2

Results of main Phase III trial according to *BRAF* mutational status

Study & Setting	Therapy		BRAF status	N pts		mPFS				mOS				ORR		
	Exp	Ctr		Exp	Ctr	Exp (mos)	Ctr (mos)	HR	p	Exp (mos)	Ctr (mos)	HR	p	Exp (%)	Ctr (%)	p
CRYSTAL*[6] *(I Line)	FOLFIRI+C	FOLFIRI	KRAS/BRAFwt	277	289	10.9	8.8	0.67	0.0013	25.1	21.6	0.83	0.0056	61.6	42.6	<0.0001
			mut	26	33	8.0	5.6	0.93	0.87	14.1	10.3	0.91	0.74	19	15	0.91
PRIME*[2] *(I Line)	FOLFOX+P	FOLFOX	RAS/BRAFwt	228	218	10.8	9.2	0.68	0.002	28.3	20.9	0.74	0.002	NA	NA	NA
			BRAF mut	24	29	6.1	5.4	0.58	0.12	10.5	9.2	0.90	0.76	NA	NA	NA
FIRE-3*[7] *(I Line)	FOLFIRI+C	FOLFIRI+B	RAS/BRAFwt	171	171	10.4	10.2	0.93	0.54	33.1	25.6	0.70	0.011	66	60	0.32
			mut	23	25	4.9	6.0	0.87	0.65	12.3	13.7	0.87	0.65	52	40	0.29
20050181*[5] *(II line)	FOLFIRI+P	FOLFIRI	wt	204	211	6.4	4.6	0.70	0.007	16.2	13.9	0.81	0.08	41	10	NA
			mut	299	294	4.8	4.0	0.86	0.14	11.8	11.1	0.91	0.34	15	13	NA
20020408*[8] *(Chemorefractory)	P	BSC	BRAF wt	115		NA	NA	0.37	<0.001	NA	NA	NA	NA	17	0	NA
			BRAF mut	9		NA	NA	0.11	0.100	NA	NA	NA	NA	0	0	NA
CO 17*[9] *(Chemorefratory	C	BSC	KRAS/BRAF wt	101	97	NA	NA			9.7	5	0.52	<0.0001	14	NA	NA
			BRAF mut	4	6	1		0.76	0.69	1.77	2.97	0.84	0.81	0	NA	NA

Exp: experimental arm; Ctr: control arm; N pts: number of patients; B: bevacizumab; C: cetuximab; P: panitumumab; BSC: best supportive care; HR: hazard ratio; mos: months; NA: not applicable; mPFS: median progression free survival; mOS: median overall survival; ORR: Overall response rate