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Review Article

RNA biomarkers in colorectal cancer

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ABSTRACT

Colorectal cancer (CRC) develops and progresses through a systematic selection for (epi) genetic alterations that drive the transformation from normal colon epithelium to adenocarcinoma. These changes affect both noncoding RNAs and mRNAs and so define the clinical behaviour of cancer cells within a distinctive host genetic and environmental context. Although earlier diagnosis and more effective treatment modalities have decreased mortality from CRC, prognostic stratification and adjuvant therapy selection after surgery remain dependent on broad descriptive classifications, opportune histological markers of poor prognosis and chemotherapy efficacy data derived from diverse CRC populations. Crucially, there is significant inter- and intra-individual variability in response to, and tolerance of, chemotherapy treatments. These limitations explain the small clinical benefit of new agents studied in contemporary phase III trials.

Molecular assays have the potential to address these constraints and there has been intense interest in the identification of clinically relevant molecular biomarkers. These must be easy to obtain and quantify and ideally represent steps in well-understood carcinogenic pathways or host-response mechanisms. Although some biomarkers can provide broad prognostic information based on CRC subtype (e.g. MSI status) or can somewhat predict response to targeted therapies (e.g. KRAS), no RNA-based biomarkers have entered routine clinical practice. This is due, in part, to the genetic heterogeneity of both patients and CRC. In addition, serious underlying issues with regards to study design, poor technical protocols, inadequate quality controls and inappropriate data analysis prevent successful translation of research results. Consequently, the identification of clinically relevant panels of biomarkers will depend not just on further advances in our understanding of CRC biology, but will need to be coupled with appropriate study designs and more suitable, standardised and transparent techniques.

1. Colorectal cancer

1.1. Introduction

Complete surgical excision of a primary tumour is the only cure for early colorectal cancer (CRC) [1]. Appropriate post-operative CRC patient management critically depends on accurate information on the extent of tumour spread. Local extent of disease and metastasis to regional lymph nodes (LNs) constitute the basis for histopathological staging, which takes into account different degrees of penetration of the primary tumour through the bowel wall (T), the degree of LN involvement (N) and the absence or presence of distant metastasis (M) [2]. The American Joint Committee on Cancer (AJCC) has condensed the TNM system into four stages, ranging from stage I defined as early cancer through to advanced metastatic stage IV disease (Fig. 1). TNM staging has been augmented by the residual tumour (R) classification, which describes the absence or presence of demonstrable residual tumour after surgery and distinguishes between potentially curative resections and primarily palliative surgical interventions: complete removal of the tumour (R0), microscopic (R1) or macroscopic (R2) residual disease.

Whilst histological indicators of cellular morphology have been the mainstay of diagnostic and prognostic assessment of CRC and are useful in providing information about tumour differentiation, aggressiveness, and risk of recurrence, they are limited in their ability to predict individual differences in clinical outcomes. For example, JCC stage II CRCs are made up of tumours with considerable heterogeneity, which results in a pathological spectrum with different 5-year disease free survival rates. This leads to inaccurate prognostication for individual patients and clinical understaging of approximately one third of stage II patients [3]. Hence a subgroup of patients with presumed early CRC harbours a minimal, but clinically significant amount of occult disease that is currently undetectable; although an emerging understanding of CRC biology is

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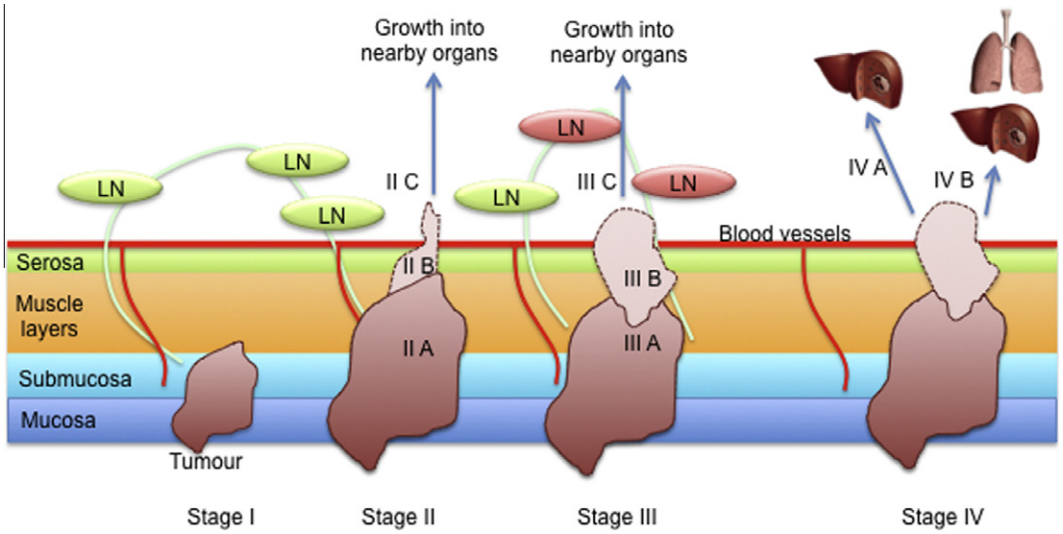

Fig. 1. CRC stages. Only some of the possible permutations are shown for stages II and III. Stage I: Cancer has spread from the mucosa of the colon wall to the muscle layer. No lymph node (LN) involvement. Stage II: Cancer has spread through the muscle layer of the colon wall to the serosa (IIA), through the serosa but not to nearby organs (IIB) or through the serosa to nearby organs (IIC). No lymph node (LN) involvement. Stage III: Cancer may have spread through the mucosa of the colon wall to the submucosa and muscle layer, and has spread to one to three nearby lymph nodes or tissues near the lymph nodes (IIIA), through the serosa but not to nearby organs (IIIB) or through the serosa to nearby organs and to one or more nearby lymph nodes or to tissues near the lymph nodes. Stage IV: The cancer may or may not have grown through the wall of the colon or rectum and has spread through the blood and lymph nodes to one (IVA) or more (IVB) parts of the body, such as the liver or lung.

beginning to identify molecular markers that may improve risk assessment and treatment choices for these patients.

The survival benefit of adjuvant chemotherapy in stage II CRC remains unproven [4], although the QUASAR study found that stage II patients treated with chemotherapy had a small but statistically significant absolute improvement in survival [5]. For patients with rectal cancers, preoperative neo-adjuvant chemoradiotherapy is utilised to decrease local recurrence rates [6], although such treatment does not compensate for incomplete surgical resection in the form of a positive circumferential resection margin [7].

Metastasis, a complex and inefficient process that has only begun to be understood in recent years, is the leading cause of CRC-related death. Clinicopathological factors are inadequate to determine the prognosis for patients diagnosed with advanced CRC, and more than one third of patients will die from progressive systemic disease. This typically develops through lymphatic vessel or capillary network intravasation that facilitates spread to nearby LN and distant organs, respectively or direct invasion of adjacent structures and transcoelomic spread within the abdominal cavity after the tumour has penetrated through the intestinal wall. Although survival has increased from 12 months with 5-FU monotherapy to around 24 months with the addition of irinotecan, oxalilaplatin and targeted drugs, several of the early steps of the metastatic cascade are not readily targetable in the clinical setting and drugs that target detectable systemic metastases often do not work in the adjuvant setting [8]. Furthermore, targeted treatment with cetuximab, bevacizumab, and panitumumab has had only a relatively small effect on survival outcomes, partly linked to the resistance of metastatic CRC to targeted therapy [9].

Improved understanding of the principal molecular networks associated with CRC is helping with the identification of new potential therapeutic agents that target individual pathways with the prospect of a high degree of biochemical specificity. Hence, in future therapies will be selected for an individual patient by assessment of their tumour and host response for tissue biomarkers predictive of disease free 5-year survival [10].

However, response rates for targeted therapy are relatively low [11] and it is not clear how effective combination-targeted therapy is [12]. Furthermore, these agents frequently have a much broader specificity than initially intended, resulting in systemic toxicity and unexpected side effects [1]. This lack of transparency makes accurate, preoperative prediction of responsiveness to chemotherapy challenging. Consequently, treatment decisions are still made almost exclusively based upon clinicopathological stage at diagnosis [13] and chemotherapeutic treatments are administered arbitrarily. As a result, this often leads to an ineffective therapy, delays the administration of a potentially useful drug, causes avoidable toxicity, encourages the development of resistant CRC subclones and incurs unnecessary cost.

Personalised medicine promises accurate prediction of an individual's predisposition towards a disease or optimised detection and management of a patient's disease in the context of an individual genetic and environmental profile. Since its realisation in clinical practice depends on the identification of safe, effective and clinically relevant biomarkers for identifying and stratifying patients [14], relevant biomarkers are the key for individualised adjuvant treatment. As previously stated, current staging strategies have difficulty with the prospective identification of patients with LN-negative tumours (AJCC stage II) who might benefit from adjuvant therapy; hence identification of molecular staging biomarkers has long been an area of research priority aimed at the identification of diagnostic biomarkers for early detection, prognostic markers for risk stratification and predictive markers associated with response to a particular therapy [15].

2. Biomarkers

Biomarkers are defined as biological substances, characteristics, or images that provide an indication of the biological state of an organism [16]. Their proper use requires an understanding of their sensitivity and specificity, how and in what contexts to use them and how to validate them properly [17]. Hence this unassuming term hides significant complexity and can refer to physiological indicators such as blood pressure, molecular markers such as expression signatures or radiological biomarkers, such as those derived from computerised tomography or magnetic resonance imaging. Unfortunately, no one biomarker is likely to have all of

the characteristics necessary to permit detection of early stage disease, facilitate prognostication or provide a robust understanding of response to cancer treatment (Fig. 2).

Cancer biomarkers have progressed from biochemical assays that measure proteins or hormones after the onset of disease to molecular assays that target disease-specific nucleic acids and promise more accurate classification, prognosis, risk stratification, treatment efficacy prediction and monitoring. In CRC a wide variety of biomarkers have been reported within the tumour itself, as well in blood and faeces, with the genetic profile of the host providing additional and essential complementary information. In addition, a range of new biomarkers, termed companion diagnostics, have been described that are designed to provide biological or clinical information that reflects the sensitivity or resistance of CRC to existing therapies and so aid clinicians in selecting the most effective therapies [18]. Typically, multigene predictors aim to stratify the risk of relapse for intermediate-stage CRC after surgical resection [19]. Gene expression profiles of normal colorectal mucosa, colorectal adenomas and different stages of CRC have identified discriminative expression signatures that mark the tumour progression sequence [20], providing a reservoir of candidate markers for the early diagnosis of high-risk colorectal adenomas, as well as potential therapeutic targets for CRC [21]. Finally, a number of biomarkers have been proposed as specific predictors of chemotherapy, radiotherapy response and, in some instances, drug toxicity [22].

3. Biomarker location

Since 95% of cases of CRC would benefit from curative surgery if diagnosis were made at an early stage of disease, early detection of colorectal adenomas at high-risk of progression to CRC is central to the aim of reducing CRC deaths. However, current screening methods are compromised by either low cost-effectiveness or limited diagnostic accuracy and detect many adenomas that will never progress to CRC [23]. Hence the importance of ongoing attempts to identify early diagnostic biomarkers, preferably present in patient stool or blood thus permitting non-invasive patient assessment. An additional benefit would be the added potential to detect more synchronous CRCs [24], which may result in additional surgical procedures and convey a poor prognosis [25]. However, as of yet no marker has progressed beyond the proof-of-principle and pilot study stage [26].

3.1. Faeces

Approximately 1.5×10^6 colonic epithelial cells can be isolated per gram of stool [27]. Hence faecal analysis constitutes a potent and noninvasive method for the detection, monitoring and management of CRC [28]. However, faeces contain nucleases as well as intrinsic substances that inhibit many molecular assays, especially PCR-based tests. Hence the molecular analysis of exfoliated epithelial cells and RNA-based markers poses a significant technical challenge [29]. RNA is highly labile and is rapidly degraded in faecal matter. Since most assays require conversion of RNA to DNA, faecal inhibitors of reverse transcriptases could affect the accuracy of the results, especially when these depend on quantifying levels of RNA. Nevertheless, there are many reports describing cancer-associated variation of RNA levels in stool samples. Most rely on the detection of one or two markers: abnormal expression of CD44 variants has been reported in the faeces of 60–70% of CRC patients before surgery, but only in 10–30% of patients after surgery [30]. Detection of cyclooxygenase 2 (COX-2) mRNA on its own [31], or in combination with matrix metalloproteinase 7 (MMP-7) mRNAs [32] has been proposed for the detection of CRC, although another report detects COX-2 mRNA in only 50% of cancer patients [33]. A recent report suggests that patients with high faecal KIAA0247 mRNA levels have a significantly greater 5-year overall survival rate, and may be associated with therapeutic benefit following administration of 5-FU [34]. However, it is rather uncertain whether these results, and those of many other studies [35], represent real differences in mRNA levels or are artifacts induced by sample preparation, differential RNA stability or inhibition of either the reverse transcription (RT) or the PCR reactions.

3.2. Blood/plasma

Haematogenous spread of CRC cells from a primary tumour is a crucial step in the metastasis cascade, and circulating tumour cells (CTCs) are considered an indicator of tumour aggressiveness [36]. Hence they may provide a potential source of cells for real-time monitoring of CRC patients through the course of their disease, enabling the detection of early dissemination of cancers, their molecular characterisation as well as monitoring treatment response or resistance. Consequently, a lot of attention has focused on the development of assays for their reliable detection and quantification.

Quantitative real-time PCR (qPCR) is the most widely used method for the detection of CTCs, but the clinical relevance of the various results reported remains uncertain. This is illustrated by a report that concludes that CK20 mRNA detection in blood samples of patients with stage AJCC II CRC identifies individuals with poor outcome [37]. This conclusion is questionable since not only is CK20 not expressed in 20% of CRC, but this is associated with higher grade carcinomas [38] and although that study did not enrich for epithelial cells, it reported that 174 blood samples from

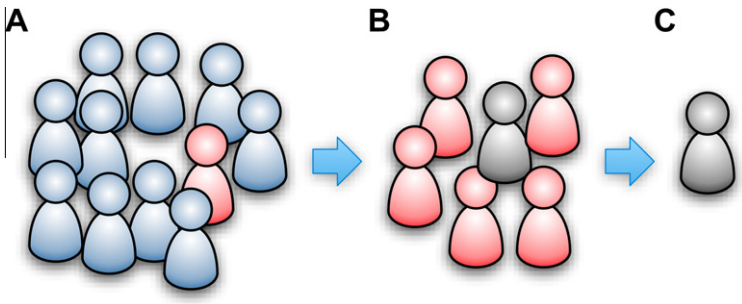

Fig. 2. The figure illustrates the difference between diagnostic, prognostic and predictive biomarkers. (A) Diagnostic biomarkers signal the risk of developing a CRC (e.g. HNPCC) or its presence. The red individual harbours an early stage cancer. (B) Prognostic biomarkers markers may be single traits or a signature of traits used to assess the risk of disease recurrence by stratifying populations with respect to the risk of disease outcome in the absence of treatment. Red individuals will relapse. (C) Predictive biomarkers, which can be either positive or negative, may be a single trait or signature of traits that identify subpopulations of patients who are most likely to respond to a given therapy and can themselves be targets for therapy. The black individual will benefit from treatment.

98 controls consistently tested negative for CK20 expression. This contradicts numerous published results, reviewed in [39], including a report that detects CK20 expression in 22% of normal healthy controls even after immunobead enrichment [40]. Similar contradictory results were obtained in two meta-analyses that used the same markers (CEA only, CEA and CK20, CK20 only or CK19 and CK20) as surrogates for circulating tumour cells (CTCs). The first looked at nine studies and concluded that disease-free survival was significantly higher in the CTC negative groups [41]. A second meta-analysis of 12 different studies concluded that CTC detection in peripheral blood is not an independent predictor of survival [42].

Similar contradictions are apparent when more than one or two markers are used. A recent review of nine studies in patients with non-metastatic CRC demonstrated that detection rates of CTCs varied from barely detectable to 57%, with seven studies claiming the presence of CTCs to be a prognostic marker of poor disease-free survival [43]. But which markers should we use? One study suggests that CEA/CK19/CK20/GCC expression identifies CTCs in CRC patients with metastatic cancers [44], another that poor survival is associated with positive detection for CEA/CK20/EGFR [45], with a third opting for telomerase reverse transcriptase/CK19/CK20/CEA mRNA expression as an independent predictor for postoperative relapse [46]. A combination of CEA/CK19/CK20/CD133 levels is reported to have prognostic significance in non-metastatic CRC patients [47], but that paper does not report any RNA quality assessment data or PCR efficiencies and all RT-qPCR data are normalised against a single unvalidated reference gene. This makes it difficult to judge whether these results are real or caused by variable RNA quality or PCR efficiencies and even if they are comparable, normalisation of human tissue biopsies against a single unvalidated reference gene has long been shown to be unreliable [168].

Another report analysing the expression of CEA/CK19/CK20/CD133/VEGF/EGFR/Survivin found that only CD133 could independently predict the survival of these patients [48]. This finding is rather surprising, given that CD133 is a marker for haematopoietic progenitor cells [49] and may or may not be a colorectal stem cell marker [50].

The most widely quoted evidence for an association between CTCs and survival comes from a prospective RT-qPCR study that has demonstrated a significant adverse impact on survival with the presence of $\geqslant 3$ CTC per 7.5 ml blood based on selection for EpCam and detection of CK19, CK20, CEA, or EGFR [51]. These results are similar to those obtained using a different method, the immunodetection-based Veridex Cell search system, which also established a cutoff of $\geqslant 3$ CTCs [52] per 7.5 ml blood. However, it is difficult to compare these methods, since they have different sensitivities, with the sensitivity of immunodetection reported to be similar to that of RT-qPCR [53] as well as significantly lower [54]. Furthermore, another study identified a cutoff of $\geqslant 2$ CTCs/7.5 ml [55], which at the very least suggests that further refinement of cell measurement is required. Finally, a recent meta-analysis concludes that detection of CTCs in peripheral blood, but not in mesenteric blood or bone marrow indicates poor prognosis [56]. However, this analysis does not scrutinise experimental protocols, RNA or assay quality assessments and omits several relevant studies. Another report identified 2/346 genes showing significantly elevated transcript levels in peripheral venous blood specimens of tumour patients when compared to the nonmalignant control group [57]. Needless to say that neither is used for the detection of CTCs in any of the above studies.

For the moment, the uncertainty with respect to immunobead detection, the absence of correlation between the level of CEA expression in tumour biopsies and that of the serum [58], and the discordance with respect to which maker to use implies that it is best to remain unconvinced about the clinical validity of these data. There is simply too wide a range of experimental protocols, criteria for sample selection, poor quality of assay design, RNA quality control and lack of transparency in reporting data that generate huge variability in reported results [59,60].

Other RNA targets being proposed as prognostic markers of one kind or another are EVI2B, ATP2A2, S100B, TM4SF3, and OLFM4 for postoperative CRC patients with unclear clinical selection criteria [61], with a subset, S100B, TM4SF3 and OLFM4 reported to correlate with liver metastasis [62]. Clearly there is an urgent need for standardised isolation and analysis techniques [63] as well as an international consensus on choice of detection method and markers [64]. Assuming the assays are detecting genuine CTCs, their clinical relevance must be verified in large-scale clinical trials [65] before their incorporation into risk stratification and clinical decision-making processes in the hospital setting [66].

3.3. Primary tumour

A new gene expression classifier for improved risk stratification of patients with AJCC stage II CRC, but not stage III, has just been published [67]. Although the independent prognostic value of the classifier was confirmed by multivariate analysis, the gene list differs from all previously published genes and gene lists. This confirms a previous finding that suggests the overlap of candidate gene lists associated with specific clinical/biological phenotypes remains disturbingly poor between studies [68]. The challenge is illustrated by a recent review of 23 independent gene expression profiling studies on CRC prognosis that identified 1475 unique, mapped genes, of which only 54 genes were reported in at least two studies and showed consistent direction in expression change between the single studies [69]. The interpretation and translation of these studies is impeded by poor experimental protocols, heterogeneous patient populations, unclear significance of the genes included in each signature, minimal external validation and non-transparent reporting of protocols and analysis methods [68,70]. Furthermore, the prognostic value of these gene signatures has not been carefully compared with that of conventional clinical and pathological risk factors [71].

One of the major limitations with the majority of available data is the small sample size each study investigates, which can result in data overfitting. This is because the number of markers queried vastly exceeds the number of patients tested, which may result in a spurious association between data and outcome, generating a model that "fits" the data by chance. Hence the choice of appropriate classification algorithm is essential for analysing these data and has led to the development of several classification algorithms [72–74]. Furthermore, the validation of any model requires that it be tested on a completely independent set of samples. Many studies struggle to find a sample set of appropriate size and comparable characteristics. For example, the identification of a set of markers able to distinguish patients with AJCC stage III CRC with no recurrence from patients with stage IV carcinoma with hepatic metastasis is based on results from nine and ten patients, respectively [75]. An entirely different-gene prognostic signature was obtained from another comparison of gene expression patterns between 41 metastatic and non-metastatic stage-matched human CRCs [76]. A 14-gene signature potentially predicting response to LV, 5-FU, and irinotecan (FOLFIRI) contains yet another gene list without any obvious association with biochemical pathways involved in CRC and is based on 21 patients [77]. Two Japanese studies have published gene signatures that predict responders to FOLFOX therapy. The first study identified a 21 gene signature, based on the differential expression between 40 responders and non-responders [78], the second one published a 14-gene signature based on 42 responders and 41 non-responders [79]. Most

impressively, not a single one of the classifier genes in either study is found in the other one.

Another limitation relates to questionable experimental protocol standards, with few reports describing adequate RNA quality, RT or PCR efficiency assessments. Furthermore, the restrictions imposed by the choice and location of oligonucleotides used in expression microarray studies have become a potential source of error since it has become clear that splicing aberrations are characteristic of cancer development [80]. This provides not just a potential source of candidate biomarkers [81], but also poses problems for the interpretation of gene expression analyses using microarrays and RT-qPCR. Finally, tumour risk and response to therapy is not just cancer-dependent, but also associated with host characteristics; hence the importance of a recent report describing a strong correlation between host immune response and a twelve immune gene-related signature associated with better patient survival independent of tumour staging, site, microsatellite instability or stability, and patient treatment, suggesting some beneficial, intra-tumoural immune cell priming [82].

A study using a combination of RT-qPCR and immunohistochemistry reports that increased expression of MMP2 in primary tumours is associated with lower overall survival, but fails to provide any clinical information on the patient group [83]. On the other hand, higher expression of MMP2, together with that of MMP 9, −11 and −14 in liver metastases has been reported as associated with a favourable response to palliative, 5-FU-based chemotherapy [84]. MMP7 is of particular interest, since its expression is confined to carcinomatous epithelium and it has been reported as an independent prognostic factor for survival in advanced CRC [85]. Of the other MMPs investigated, MMP21 may be an independent prognostic factor in patients with AJCC stage II as well as stage III CRC [86] and MMP13 has been reported as associated with postoperative relapse [87].

The value of molecular markers to predict rectal cancer response to preoperative chemoradiation is equally unclear [88,89]. Although higher expression of MMP9 alone in epithelium and lower expression in stromal cells has been reported as a prognostic marker for overall survival [90], a recent serial analysis of gene expression (SAGE) and RT-qPCR analysis has identified a thirteen-gene signature from AJCC stage II/III patients that claims to predict preoperative chemoradiotherapy response and outcome in rectal cancers with an overall accuracy of 76% [91]. Again it is instructive that this gene signature does not include MMP9.

3.4. miRNA

MicroRNAs (miRNAs) are a class of endogenous short (~22 nucleotides) noncoding RNAs capable of regulating the expression of protein-coding genes at the post-transcriptional level by cleaving target mRNAs and/or repressing their translation. They appear to be remarkably stable and resistant to RNases and have been isolated from stool, sputum, serum and plasma, allowing a wide range of applications in clinical research. Cancer is associated with frequent aberrant expression of miRNAs, which are capable of functioning as oncogenes or tumour suppressors to modulate multiple oncogenic cellular processes. miRNA expression levels in tumours are increasingly acknowledged as potential prognostic biomarker for CRC: miRNA expression plays an important role in CRC development and prognosis, with miRNAs regulating all the major pathways deregulated in CRC [92]. miRs-135a and 135b regulate the critical wnt pathway through their downregulation of APC gene expression, which activates the transcriptional cofactor function of β-catenin [93].

From a practical point of view miRNAs are very stable in archival material and can be retrieved efficiently from samples as old as 28 years [94]. However, as with mRNA-based biomarkers, there have been numerous incomplete and contradictory reports about their role and until issues of RNA and assay quality assessment, as well as transparent and complete reporting of experimental details are resolved, the translatability of these results remains uncertain [95].

There are many reports suggesting prognostic roles for one or two biomarkers. Low levels of miR-148a and miR-152 may be found in advanced stages of disease [96] and downregulated miR-22 [97], miR375 [98] and miR-143 [99] may predict poor prognosis, with a significant correlation between miR-145 expression and rectal cancer regression also reported [100]. Conversely, expression of miRNA-93 may reduce CRC recurrence [101], whereas elevated miR-21 levels have been found in patients unresponsive to fluorouracil chemotherapy and with poor long-term survival [102]. High levels of miR-31 may be associated with distant metastases and let-7a is overexpressed only in metastasis but not in primary cancer.

Multiple miRNA targets have also been identified, with a prognostic signature of miR 21,135a, 335, 206 and let-7a proposed for the detection of the presence of metastases [103], although it only has a specificity of 87% and sensitivity of 76%. The presence of a KRAS mutation is associated with dysregulation of several miRNAs that target genes involved in apoptosis and proliferation [104]. Furthermore, a colon miRNA signature comprising eleven overexpressed and eight underexpressed miRNAs may be involved in regulation of stem cell differentiation and thus provide potential cancer stem cell therapy targets [105].

Relatively little work has been done on the significance of circulating miRNA in CRC [106] and there is little consistency in the reported results. miR-17-3p and miR-92a levels may be significantly higher in the plasma and cancer samples of CRC patients, with plasma levels significantly reduced after surgery [107]. Another study reports that that high levels of miR-221 may be a potential marker for diagnosis and prognosis of CRC [108]; in contrast a third study reports that plasma levels of miR-29a and miR-92a, but not miR-17-3 or miR-221 have diagnostic value [109]. Although another study also found that serum miR-29a levels were significantly higher in patients with liver metastases [110], the sensitivity and specificity of that assay was only 75%. Yet another study found that circulating miR-34a levels are reduced in CRC patients [111].

Unsurprisingly, several studies have attempted to use miRNAs from faeces as screening and prognostic markers for CRC although, as is now usual, there is no agreement between the studies. An analysis of miRNA expression of exfoliated colonocytes identified the miR-17-92 cluster and miR-135 as being significantly upregulated in CRC tissues compared with normal colon, although the overall sensitivity and specificity was only 74.1% and 79.0%, respectively [112]. In contrast, another study identified increased expression of miR-21 and miR-106a in the stool of CRC patients [113]. Intriguingly they were higher in patients with adenomas and tended to decrease in cancer. Another study suggested down-regulation of fecal miR-143 and miR-145 as potential markers for CRC [114].miRNAs also have potential therapeutic applications, since synthetic oligonucleotide antagomirs that inhibit overexpressed miRNA such as miR-135 can result in an increased expression of APC in CRC cell cultures and miRNA mimics can compensate for downregulated miRNAs such as miR-143 and miR-145, leading to decreased cancer cell proliferation *in vitro* [92].

4. Practical limitations

The excitement about biomarkers originating from research laboratories must be balanced by a critical review of the available

evidence before establishing their use in clinical decision-making [115]. As has become clear, many of the studies reporting the discovery of biomarkers useful for CRC management are retrospective, involve small series of patients and are unable to predict disease progression accurately with clinically adequate resolution and reproducibility [116]. Sample size is important as small studies can give inflated, over-promising results as a result of selection bias [117]. While small study populations are likely to be more homogeneous and thus molecular classifiers may be more efficient, they are frequently underpowered and thus unable to discriminate informative molecular signatures and may incorrectly reach negative conclusions. Study endpoints are an additional critical aspect of oncologic clinical trials that require careful attention when assessing the relevance of biomarkers and the efficacy of treatment [118]. As a consequence, although a combinatorial approach to molecular prognostics, similar to the that established for breast cancer patients, may have significance and be used in future for CRC patient management [119], currently research has failed to yield consistent sets of externally validated markers [120] that clinicians could use for clinical decision making [121].

Even when concerted efforts are made to minimise the number of variables, the data themselves are so vast that they can contribute significant background noise. Clearly, it is essential to combine and integrate molecular data with other sources of genomic, proteomic, biomedical and clinical data from patient records.

The findings of many studies are contradictory and the current reality is that a single genetic marker, KRAS gene, has made the transition into clinical practice in the case of EGFR-targeted therapy for metastatic disease [122]. Disconcertingly, even though current evidence and expert consensus does not support the clinical validity of RNA-based biomarkers, there are commercial tests that appear to be marketed as useful assays supporting the clinical decision-making process for individual cancer patients [15]. So although both the Oncotype DX Colon and ColoPrint gene signatures may provide prognostic information about risk of recurrence, neither assay has demonstrated the ability to predict which patients will benefit from adjuvant chemotherapy, a primary goal of personalised oncological medicine [4].

Finally, there is another important, rarely publicised reason for the observed discrepancies among peer-reviewed publications. Recent years have seen a significant increase in the number of retracted publications, many reporting the use of molecular techniques to link expression signatures, clinicopathological features and individualised therapy in various cancers, including CRC. The most common reason for these retractions is error [123,124] and deliberate fraud [125]. These retractions are complemented by numerous corrections that are frequently sufficiently extensive to warrant a retraction as well as rebuttals that point out mistakes in publications. Furthermore, the frequency of retraction shows a strong correlation with the journal impact factor [126]. Worryingly, rebuttals have no effect on the number of times that publication is cited, and even then the citing papers on average have neutral views of the original article, or believe that the rebuttal agreed with it [127]. As a result, the peer-reviewed literature is plagued by contradictory, irrelevant, wrong or fraudulent publications that combine to pose a challenge to the integrity of the scientific literature, with serious consequences not just for basic research, but potentially calamitous implications for drug development and disease monitoring [128]. Indeed, this problem is abundantly clear to any reader of the literature concerning molecular biomarkers, as has been referred to repeatedly in the course of this review.

4.1. Biological variability

The biological variability that is characteristic of human beings introduces an important confounding issue into the identification of universal molecular biomarkers:

- Colorectal tissue consists of individual cells from a variety of lineages that reside in a changeable natural environment and interact as coordinated and dynamic partners.
- Genetic and epigenetic differences generate unpredictable and unique genomic backgrounds.
- The internal environment of each cell is highly stochastic with constantly changing concentrations of metabolites, RNA, regulatory molecules and proteins [129].

This results in significant differences in gene expression patterns between individual cells [130] that create variable behaviour patterns [131] and must not be ignored [132]. It must also be remembered that CRC biopsies are composed of epithelial cells as well as numerous other cell types. Variable proportions of these non-epithelial and non-malignant cells could lead to inconsistent and biologically inaccurate gene expression patterns. Although analysis of microdissected cell populations partly addresses these problems [133], this creates its own disadvantages: a significant proportion of metastasis-associated signatures appear to be derived from the nonepithelial component of the tumour and microdissection would result in the loss of that component [134]. The case for considering expression profiles of both epithelial and stromal cells is further strengthened by strong evidence that the interaction between malignant cells and (myo)-fibroblasts [135] as well as macrophages [136] in the tumour microenvironment modulates the biological behaviour of CRC All these factors may generate noise that could mask expression patterns relevant for outcome prediction.

Data interpretation following expression profiling experiments is further complicated by our patchy understanding of the complexity of biological systems, illustrated by wide baseline variability of RNA levels in individual cells and tissues due to normal *in vivo* degradation [39], transcript splicing that affects most human genes [137] and plays an important role in cancer pathology [138], differences in allelic expression among autosomal non-imprinted genes in animals [139], the discovery of novel RNA species [140] and new findings in relation to the regulatory roles of RNA [141]. Appropriate biological replication is essential if data are to be valid in the context of a large population from which the subjects were sampled, rather than only for the particular individuals considered in the experiment [142]. Since biological variability is larger than technical variation, increasing biological replication usually translates into more effective gains in power. However, increasing sample size generally leads to increased cost and time requirements to perform the experiments. In addition, some biological replication cannot be increased, e.g. when comparing large numbers of healthy individuals with a limited number of patients with a particular disease. Clearly, biologically relevant data interpretation requires the development of dedicated experimental protocols and analytical procedures [143] as well as incorporation into more realistic statistical models to allow biologically relevant data interpretation [144]. One consequence of this is the need for movement towards a quantitative description of complex biological systems involving the interaction of many components, rather than a narrow focus on a description of single biomolecules or even pathways and their interaction with other individual molecules or pathways.

Finally, host genetics plays a critical and increasingly recognised role in determining tumour behaviour as well as patient response to adjuvant therapies. In practice this means that malignancy is

influenced not just by environmental stimuli, or multiple genetic and epigenetic events that arise within the malignant epithelium, but also by the genetic background of the host [145]. In addition, there is an important contribution from the tumour microenvironment and metastasis can be modified by stromal events. Consequently it is plausible that multiple signatures could be a consequence of the multiple pathways through which CRC metastases arise [146], as demonstrated by the fact that LN and liver metastases from the same patient do not always show the same genetic aberrations [147]. This implies that the accuracy of multigenic classifiers might be improved if their selection were based on better understanding of the underlying tumour biology. The finding of a molecular signature in primary cancers predictive of metastasis [134] suggests that the metastatic potential of many cancers is encoded in the bulk of the primary tumour and raises the expectation that this will result in a prognostic application. Nonetheless, it is notable that CRCs were not included in the demonstration of clinical utility for their metastasis-associated signature, although another study does describe such a signature for AJCC stage III CRC patients [148]. Interestingly, this study also suggests that detection of the expression of a single mRNA, the RAS homologue RHOA, can identify a subset of patients that may benefit from adjuvant chemotherapy. However, this depended on selecting appropriate thresholds for mRNA levels, with the associated problems this entails [59].

4.2. Technical variability

A second major cause for the scarce translation of molecular biomarkers into clinical practice is not just a lack of quantitative techniques [122], but an immense technical variability that derives from sampling, assay design and measurement errors that results in a lack of definition, adequate validation, and hence easy implementation of quantitative data [121]. This is readily illustrated by a recent report demonstrating that the use of formalin-fixed, paraffin-embedded samples to classify CRCs according to their methylation status generates unsatisfactory and non reproducible bisulfite conversions leading to random results for methylation levels [149].

4.2.1. Microarrays

There are several technical limitations that have the potential to introduce uncertainty into the reliability of DNA microarray measurements and whilst the Minimum Information About a Microarray Experiment (MIAME) guidelines [150] describe the minimum information required to enable microarray data interpretation and independent verification, they do not endorse technical standards. Consequently, microarray experiments continue to be characterised by numerous potential liabilities: hybridisation conditions for different genes are variable [151], misinterpretation of gene expression data that occurs when RNA quantities are inappropriately normalised [152], inappropriate control samples that limit the validity of the results [153] and limitations of analysis programs [154]. As a result accurate measurements of expression levels and the reliable detection of low abundance genes continue to represent significant challenges for microarray technology. Hence, whilst the direction of change indicated by microarray experiments may be consistent, the magnitude of gene expression changes is much less reliable [68] This is one reason why RT-qPCR-based high throughput massively parallel quantification systems, as well as next generation sequencing systems are likely to supplant microarrays for truly quantitative measurements (but see below).

Furthermore, there is little consistency between studies used to predict clinical outcome [155]. This was demonstrated by the lack of association between the expression profiles of several cell cycle regulatory or proliferation-related markers previously correlated with prognostic relevance and disease-free survival in LN-negative rectal cancers treated by surgery alone [156]. It is apparent that single microarray data are prone to false results [157] and that different studies result in different gene lists; indeed different gene-selection methods can lead to strikingly different gene lists from the same experiment [158]. It is hoped that the establishment of gene co-expression networks for functionally related genes will improve the biological validity of microarray data, exemplified by a study that combined independent datasets on different types of cancer to explore transcriptional changes in terms of gene interactions rather than at the level of individual genes [159]. Two distinct networks were able to detect biological changes and identify gene groups whose co-regulation might contribute to malignant transformation.

4.2.2. RT-qPCR

RT-qPCR technology provides a textbook example of the technical problems associated with technologies commonly assumed to be mature by clinicians, but which are actually full of technical pitfalls. The ubiquity of RT-qPCR has resulted in an abundance of protocols that differ at every stage of the experimental workflow and provide ample scope for the introduction of inconsistencies that are responsible for the many contradictory results reported in the scientific literature. Discrepancies are further magnified by experiment- and individual-specific variation, since even though tissues or targets may be seemingly the same, experimental samples, protocols and data analysis methods inevitably differ. Other inconsistencies arise because the investigator has a choice of distinct instruments, a wide range of enzymes that are further adapted by manufacturer-specific reaction buffers and data analysis software based on different statistical methodology [128].

Poor experimental design is inherently associated with technical variability: inappropriate underlying assumptions generate results that may be biologically or clinically of little consequence and have negligible translational relevance [160]. This source of variability is prompted by experimental designs employing false assumptions, deficient technologies, inappropriate sampling procedures, inconsistent use of controls, incorrect methods of normalisation, unsound data analysis procedures and misdirected statistical methodologies [161]. As discussed earlier, there are numerous instances of publications reporting one observation and being contradicted by others reporting opposite results, impeding the translation of qPCR technology into clinical practice.

The key to minimising variability is careful attention to [162] as well as assiduous reporting of [128] experimental detail. Regrettably, the vast majority of publications do not provide sufficient information to allow the reader to infer whether published data support the conclusions of the publications [163]. In principle, given sufficiently comprehensive information, any reader comparing discordant published results should be able to discern which ones are likely to be caused by flaws in experimental design, execution or interpretation. In practice, this essential detail is usually not available and detailed examination of discordant data is either not possible or requires the investment of an inordinate amount of effort. The growing consensus around the need to improve published information with relevant experimental detail, as well as issues relating to pre- and post-assay parameters has resulted in the publication of the "Minimum Information for Publication of Quantitative Real-Time PCR Experiments" (MIQE) guidelines [164], with a recent amendment clarifying the disclosure of primer sequences [165]. The last year has seen a rapid adoption of these guidelines and their implementation by clinical researchers should encourage detailed auditing of experimental detail, data analysis and reporting principles and so contribute to the publication of more clinically relevant, and hence translatable results.

5. Conclusions and outlook

CRC arises from complex, variable and patient-specific interactions between genetic, epigenetic and environmental factors (Fig. 3). The development, improvement and increased sophistication of a wide range of molecular tools has accelerated the transition to large-scale systemic approaches for the study of this disease, making it possible to analyse the expression patterns associated with its molecular pathogenesis. As a consequence, the last 20 years have witnessed a remarkable increase in knowledge of the CRC transcriptome. The identification of critical molecular pathways associated with CRC tumourigenesis and metastasis has demonstrated the feasibility of achieving better outcome prediction by combining large-scale molecular analyses with classic morphologic and clinical methods of staging and grading cancer. This is driving the pursuit for a change of emphasis of tumour classification from morphological to molecular markers and has led to the identification of numerous potential prognostic biomarkers.

Nevertheless, although numerous individual markers and several expression profiles have been reported as independent predictors of disease outcome, none have been universally validated and they have not yet been adopted into routine clinical decision-making [10]. Studies often generate vast amounts of information without any clear evidence that this is any more relevant than available strategies for practical patient management. Hence, despite the introduction of targeted drugs for the treatment of advanced CRC and improved overall survival for non-resectable disease, cure rates remain low. Currently KRAS is the only sufficiently validated predictive molecular marker for anti-EGFR directed therapy, with the predictive value of BRAF mutations still unclear. Even so, there are currently no US Food and Drug Administration-approved assays for the detection of KRAS mutations [166].

There are numerous studies underway aimed at incorporating putative predictive molecular markers into the clinical decision making process, and it seems safe to suggest that the use of molecular markers in routine clinical practice will increase as more markers are identified and validated. Furthermore, technical progress continues apace, with 2012 witnessing the introduction of new sequencings instruments that enable a whole human genome to be sequenced at a run cost of $1000. Together with the identification of new, selective inhibitors of the signalling pathways critical to CRC and the development of innovative technologies for the simultaneous detection of biomarkers and therapeutic drugs, this will lead to the enhanced potential for targeted disease management in the individual patient.

However, a lot remains to be done. There are numerous technical issues that need to be addressed, not least the need to develop a range of software tools to incorporate patients' genomic information into the clinical decision making process. It is essential that technical variability is minimised by using operating procedures that implement rigorous standards at each step of a study, especially with regard to careful experimental quality control with associated quality metrics [167]. This is of particular importance where studies aim to correlate particular clinical outcomes with mRNA or miRNA expression profiles changes. Extensive validation of expression profiles on external sets is essential and it is important that clinical studies are more carefully designed with stringent criteria for assigning outcomes to samples. There is no substitute for disease-free survival; hence these clinical studies are likely to involve long-term prospective trials in order to determine whether

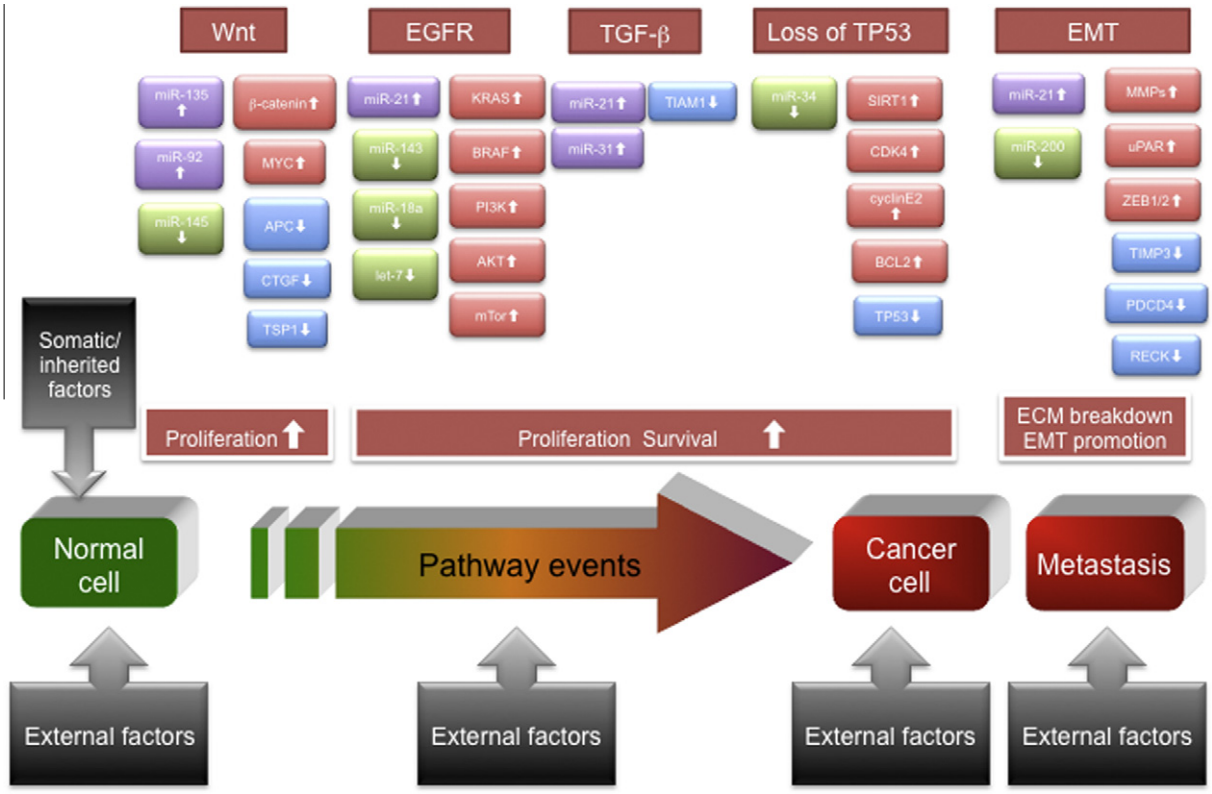

Fig. 3. CRC tumourigenesis. Progression from normal epithelium through adenoma and CRC to metastasis is characterised by accumulated abnormalities of individual miRNAs (purple and green) and mRNAs (blue and red) affecting key signalling pathways. ECM (extracellular matrix), EMT (epithelial-mesenchymal transition). Carcinogenesis progresses by several pathways, usually characterised by early acquisition of APC mutations that lead to deregulated wnt signalling. Some tumours harbour frequent activating mutations of the KRAS oncogene and inactivating TP53 mutations. Others frequently acquire BRAF mutations and are not associated TP53 mutations.

expression-based cancer profiling is equal to, or better than conventional methods. Finally, we would like to remind researchers and clinicians alike that every new molecular technique shows tremendous promise until subjected to the rigorous evaluation of real-life clinical diagnostics.

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