Consensus molecular subtypes and the evolution of precision medicine in colorectal cancer

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Abstract | Critical driver genomic events in colorectal cancer have been shown to affect the response to targeted agents that were initially developed under the 'one gene, one drug' paradigm of precision medicine. Our current knowledge of the complexity of the cancer genome, clonal evolution patterns under treatment pressure and pharmacodynamic effects of target inhibition support the transition from a one gene, one drug approach to a 'multi-gene, multi-drug' model when making therapeutic decisions. Better characterization of the transcriptomic subtypes of colorectal cancer, encompassing tumour, stromal and immune components, has revealed convergent pathway dependencies that mandate a 'multi-molecular' perspective for the development of therapies to treat this disease.

Early-stage
Tumours that remain localized and have not spread to other parts of the body.

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Colorectal cancer (CRC) is a leading cause of cancer-related death worldwide[1,2]. However, mortality associated with a CRC diagnosis has declined progressively in the past decades[2,3], which can be attributed to cancer screening programmes, the standardization of preoperative and postoperative care, improved surgical techniques and the availability of more-effective systemic therapies for early-stage and advanced-stage disease[3]. Fluoropyrimidines, oxaliplatin and irinotecan represent the chemotherapy backbones for the treatment of metastatic CRC, and their sequential administration results in median overall survival ranging from 18 to 20 months[4]. After targeted agents such as vascular endothelial growth factor (VEGF) inhibitors and epidermal growth factor receptor (EGFR) inhibitors were added to the therapeutic armamentarium in combinations with the above chemotherapies, median survival rose to 30 months[4]. Various anti-angiogenic agents are approved for clinical use, including the monoclonal antibodies (mAbs) bevacizumab and ramucirumab, the recombinant fusion protein aflibercept and the multi-kinase inhibitor regorafenib. On the one hand, robust predictive biomarkers for anti-angiogenic treatment prioritization have not yet been identified. On the other hand, the selection of patients for anti-EGFR therapy with the mAbs cetuximab and panitumumab is based on the absence of MAPK gene mutations, namely *KRAS* and *NRAS* activating events, which confer innate resistance[5].

The evolution of targeted therapies in CRC has been characterized by the slow and gradual recognition of a number of biomarkers that predict negative responses to anti-EGFR agents. But recent advances in our understanding of the genomic and transcriptomic subtypes of CRC — with unique clonal, stromal and immune dependencies — and the recognition that tumours evolve under pressure from treatment have positively influenced biomarker–drug co-development. In this Review, we discuss the current trends of translational research in CRC and patient stratification strategies for 'omics'-guided therapies. We propose an integrative classification system that links molecular features to targeted drugs, re-examine previous successes and failures, and envision the future of precision medicine in CRC.

Molecular understanding of CRC

Driver events, genomic and epigenomic subtypes. CRC was one of the first solid tumours to be molecularly characterized, and several genes and pathways were implicated in tumour initiation and growth[6]. The model of progressive step-wise accumulation of genetic and epigenetic events leading to adenoma and carcinoma formation[7–9] described by Vogelstein and colleagues provided insights into the role of 'driver' alterations in tumour suppressor genes (such as adenomatous polyposis coli (*APC*), *TP53* and SMAD family member 4 (*SMAD4*)) and oncogenes (such as *KRAS* and PI3K catalytic subunit-α (*PIK3CA*)) that confer selective growth advantages and give rise to CRC progression. The non-random

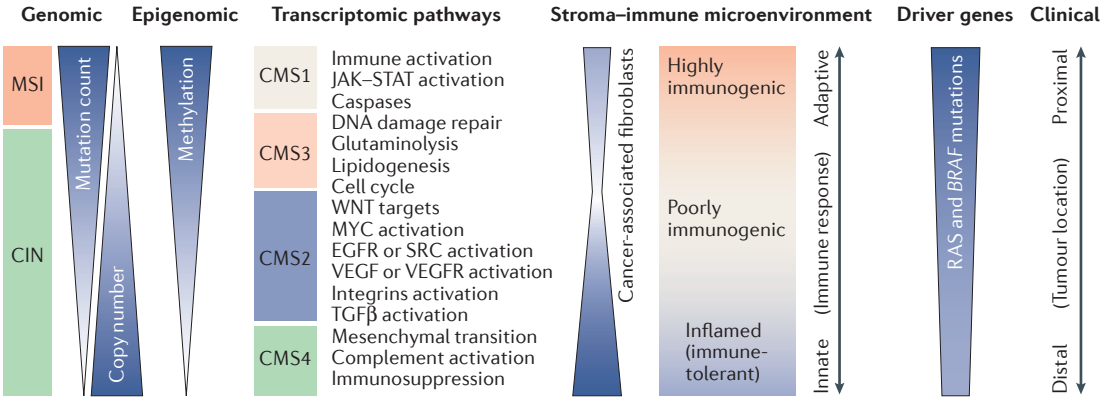

Figure 1 | **Schematic representation of CRC subtypes.** Microsatellite instability (MSI) is linked to hypermutation, hypermethylation, immune infiltration, activation of RAS, *BRAF* mutations, and locations in the proximal colon. Tumours with chromosomal instability (CIN) are more heterogeneous at the gene-expression level, showing a spectrum of pathway activation ranging from epithelial canonical (consensus molecular subtype 2 (CMS2)) to mesenchymal (CMS4). Tumours with CIN are mainly diagnosed in left colon or rectum, and their microenvironment is either poorly immunogenic or inflamed, with marked stromal infiltration. A subset of CRC tumours enriched for RAS mutations has strong metabolic adaptation (CMS3) and intermediate levels of mutation, methylation and copy number events. EGFR, epidermal growth factor receptor; JAK, Janus kinase; STAT, signal transducer and activator of transcription; TGFβ, transforming growth factor-β; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

accumulation of gene mutations initiates colorectal carcinogenesis by deregulating pathways that modulate cellular differentiation, proliferation and apoptosis[8]. Genomic studies have also shown that alterations in the WNT–β-catenin, transforming growth factor-β (TGFβ), EGFR and downstream MAPK and PI3K signalling pathways are nearly ubiquitous events in CRC[10–12].

Moreover, genome-editing technology has been used to show that a colon organoid engineered to express all *APC*, *TP53*, *SMAD4*, *KRAS* and *PIK3CA* mutations can grow independently of microenvironment factors, indicating that mutations in these genes are sufficient to initiate tumour progression[13]. However, it was only when chromosomal instability (CIN) co-occurred with driver mutations that tumours became invasive and formed macrometastasis when injected into mice[13]. Indeed, imbalances in chromosome number (aneuploidy) and loss of heterozygosity are seen in 85% of invasive CRC tumours[11] (FIG. 1). CIN results from defects in chromosomal segregation, telomere stability and the DNA damage response, and mutations in *TP53* and other checkpoint genes have a permissive role[14]. Alternatively, 15% of early-stage colorectal tumours have a defective DNA mismatch repair system (caused by inactivation of mutL homologue 1 (*MLH1*), *MLH3*, mutS homologue 2 (*MSH2*), *MSH3*, *MSH6* or PMS1 homologue 2 (*PMS2*)) as the dominant genomic feature, giving rise to hypermutation and microsatellite instability (MSI)[11]. Epigenomic studies have shown that tumours with MSI have a high CpG island methylation phenotype (CIMP^{hi}) and exhibit hypermethylation of key genes implicated in tumour development, such as *MLH1* silencing in cases with a sporadic background[15]. Although conventional adenomas share a range of molecular features with chromosomally instable CRCs, CIMP^{hi} and MSI are initial driving forces in sessile serrated adenomas[16].

The sequence and pattern of genetic and epigenetic events seems to differ in tumours with CIN (which are generally non-hypermutated) and tumours with MSI (which are typically hypermutated), and there is evidence of convergence in deregulation of the pathways described above (FIG. 2). For example, activation of the WNT–β-catenin pathway is mainly driven by *APC* mutations in non-hypermutated samples[11], whereas ring finger protein 43 (*RNF43*) mutations and R-spondin (*RSPO*) family fusions are strongly enriched in hypermutated CRC[12,17]. *TP53* loss and mutations in the cell cycle checkpoint and DNA damage response kinase ataxia-telangiectasia mutated (*ATM*) also have a mutually exclusive pattern and are predominant in non-hypermutated and hypermutated samples, respectively[11]. *KRAS* mutations are commonly seen across disease subsets, but *BRAF*^{V600E} mutations are overrepresented in tumours with MSI and CIMP^{hi} (REF. 11). Interestingly, somatic mutations in DNA polymerase ε (encoded by *POLE*) have been reported to account for the highest mutation rates in CRC, but those tumours frequently lack MSI, CIMP^{hi} or *MLH1* hypermethylation[11]. Activating kinase fusion events involving anaplastic lymphoma kinase (*ALK*), neurotrophic receptor tyrosine kinase 1 (*NTRK1*), *NTRK2*, *NTRK3* or *RET* are extremely rare in CRC and do not seem to cluster in a particular genomic or epigenomic subtype of the disease[18–20].

Consensus transcriptomic subtypes. Gene expression is intimately linked to cellular phenotype and tumour behaviour, and has been extensively used to identify biologically homogeneous subtypes of a disease. In CRC, an international effort dedicated to sharing large-scale data and coordinating analytics compared six independent transcriptomic-based subtyping systems[21–27]. This resulted in a consensus molecular classification that enables the categorization of most tumours into one of

Advanced-stage
Tumours that became metastatic and have spread to other parts of the body.

Sporadic background
Tumours that have no identifiable inherited gene involved in the carcinogenesis process.

Sessile serrated adenomas
Pre-malignant flat (or sessile) polyps, predominantly seen in the right side of the colon. They have been identified as the main precursor lesion in the serrated carcinogenesis process.

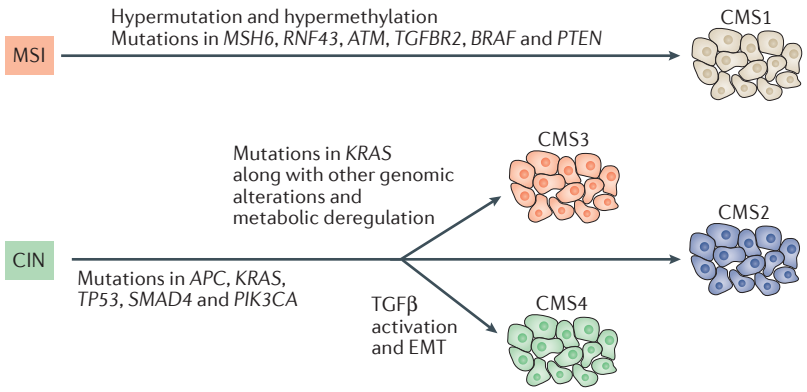

Figure 2 | **Colorectal carcinogenesis and transcriptomic subtypes.** Potential routes of colorectal adenoma–carcinoma progression with accumulation of genomic and epigenomic alterations. Whereas tumours with microsatellite instability (MSI), mostly consensus molecular subtype 1 (CMS1), have a distinctive pattern associated with hypermutation and hypermethylation, tumours with chromosomal instability (CIN), mostly CMS2–4, develop through the traditional model proposed by Vogelstein[7]. A shift from canonical CMS2 carcinogenesis to CMS3 is thought to occur early, with a unique combination of *KRAS* mutations and copy number events causing metabolic deregulation as the dominant feature at the gene-expression level. In CMS4 tumours, transforming growth factor-β (TGFβ) activation from a stromal-enriched inflamed microenvironment functions as a major driver in epithelial–mesenchymal transition (EMT). *APC*, adenomatous polyposis coli; *ATM*, ataxia-telangiectasia mutated; *MSH6*, mutS homologue 6; *PIK3CA*, PI3K catalytic subunit-α; *RNF43*, ring finger protein 43; *SMAD4*, SMAD family member 4; *TGFBR2*, TGFβ receptor 2.

four robust subtypes[28]. The four consensus molecular subtype (CMS) groups (FIG. 1) represent the current best description of CRC heterogeneity at the gene-expression level, but further refinement in disease classification, with intra-CMS subgroups and better characterization of samples with mixed phenotypes, is likely to emerge in the future.

Comprehensive correlative analyses with well-defined genomic and epigenomic CRC features enabled deeper understanding of the biological characteristics of each CMS. First, most tumours with MSI cluster in the CMS1 group (MSI immune subtype, 14% of early-stage tumours), which is characterized by hypermutation, hypermethylation, enrichment for $BRAF^{V600E}$ mutations and strong infiltration of the tumour microenvironment with immune cells, particularly $CD8^+$ cytotoxic T lymphocytes (CTLs), $CD4^+$ T helper 1 (T_H1) cells and natural killer (NK) cells. Next, tumours with CIN can be subclassified into three groups on the basis of gene expression signals: CMS2 (canonical subtype, 37% of early-stage tumours); CMS3 (metabolic subtype, 13% of early-stage tumours); and CMS4 (mesenchymal subtype, 23% of early-stage tumours). CMS2 and CMS4 cannot be distinguished by their somatic copy number alteration patterns and mutation spectrum, with both groups presenting microsatellite stability (MSS) and low levels of gene hypermethylation[28]. However, CMS2 epithelial tumours have marked upregulation of WNT and MYC downstream targets, higher expression of the oncogenes *EGFR*, *ERBB2* (also known as *HER2*), insulin-like growth factor 2 (*IGF2*), insulin receptor substrate 2 (*IRS2*) and transcription factor hepatocyte nuclear factor 4α (*HNF4A*), as well as cyclins[28]. Conversely, CMS4 tumours are characterized by activation of pathways related to epithelial–mesenchymal transition (EMT) and stemness, such as TGFβ and integrins, and show marked overexpression of proteins implicated in extracellular matrix remodelling and complement signalling[28]. CMS4 tumours exert a proangiogenic and stromagenic influence on the microenvironment. Indeed, signalling activation in tumours with a mesenchymal phenotype is mostly derived from prominent stromal cell infiltration of adjacent cancer tissue, particularly cancer-associated fibroblasts (CAFs)[29,30]. Notably, the striking differences in pathway activation between CMS2 and CMS4 tumours translate into significantly higher risk of distant relapse and death for patients diagnosed with early-stage CMS4 mesenchymal CRC[28]. Last, CMS3 tumours have a distinctive global genomic and epigenomic profile as compared with other CIN groups, with consistently fewer copy number alterations. In fact, up to 30% of the samples of CMS3 tumours present with MSI, hypermutation and intermediate levels of gene hypermethylation. The dominant feature at the pathway level of CMS3 epithelial tumours is metabolic reprogramming, including activation of glutaminolysis and lipidogenesis. In addition, CMS3 tumours are enriched for *KRAS*-activating mutations, which have been linked to prominent metabolic adaptation in CRC[31] and other malignancies[32–36].

Although the key molecular alterations of each transcriptomic subtype are potential drivers of initiation and growth of these tumours, the cells of origin of individual CMS groups have not yet been defined. Using gene-set enrichment analysis, signatures specific for lower and upper colon crypt compartments have been linked to CMS2 and CMS3, respectively[21,26,28]. However, it is unlikely that a specific cell phenotype is maintained after oncogenic transformation, considering the plasticity between colonic stem cells and more differentiated intestinal cells[37]. Conversely, there is clear evidence from the study of pre-malignant lesions that development drivers, particularly TGFβ activation, may play an important part in carcinogenesis. Using human organoid cultures and genome editing technology, investigators have shown that the genetic background of pre-malignant lesions dictates the dominating response to TGFβ, changing it from a largely apoptotic response in WNT-pathway-activated tubular adenomas to a dominant EMT response in $BRAF^{V600E}$-mutated sessile serrated adenomas[38]. Indeed, depending on the level of expression of TGFβ in the microenvironment, sessile serrated adenomas could progress to either poor-prognosis CMS4 tumours (high TGFβ signalling) or good-prognosis CMS1 tumours (low TGFβ signalling)[38]. CMS classification of tubular adenomas showed a link with canonical CMS2 and metabolic CMS3 groups[38]. At histopathological assessment, adenocarcinomas of each transcriptomic subtype may display dominant features, including a desmoplastic reaction with high stroma in CMS4, solid and/or trabecular or mucinous features in CMS1, complex tubular structure in CMS2 and papillary morphology in CMS3 (REF. 21). However, these architectural patterns are not diagnostic of each CMS group.

Desmoplastic reaction
At histopathological examination, pervasive growth of dense fibrous connective tissue around the tumour.

Trabecular
At histopathological examination, tumours composed of cells structured in a nested pattern.

Mucinous
At histopathological examination, tumours characterized by abundant extracellular accumulation of mucus bound to neoplastic epithelium or stroma.

Papillary
At histopathological examination, tumours demonstrating prominent papillae with fibrovascular cores.

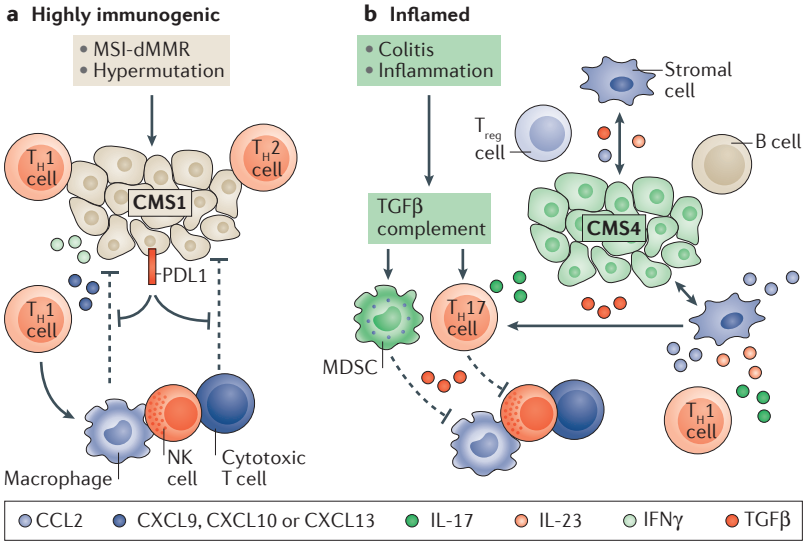

Figure 3 | **Immune characterization of colorectal cancer in light of genomic and transcriptomic subtypes. a** | In hypermutated samples of consensus molecular subtype 1 (CMS1) microsatellite instability (MSI) immune subtype, highly immunogenic neoepitopes activate an immune microenvironment infiltrated with adaptive cytotoxic cells, which is counterbalanced by the expression of checkpoint inhibitors, such as programmed cell death protein 1 ligand 1 (PDL1). **b** | Chronic inflammation, potentially linked to intestinal microbiota and colitis[51], triggers an innate immune reaction that drives tumour growth by allowing cancer cells to evade immune attack. In the inflamed microenvironment of CMS4 mesenchymal colorectal cancer, stromal cells interact with cancer cells through immunosuppressive chemokines that inhibit cytotoxic immune cells and promote the proliferation of myeloid-derived suppressor cells (MDSCs), B cells and regulatory T (T_{reg}) cells. CCL2, C-C motif chemokine ligand 2; CXCL, C-X-C motif chemokine ligand; dMMR, defective mismatch repair; IFNγ, interferon-γ; IL, interleukin; NK, natural killer; TGFβ, transforming growth factor-β; T_H, T helper.

Evolving immune subtypes. Galon and colleagues first demonstrated the relevance of specific immune signatures in the prognosis of early-stage CRC[39–41]. High lymphocyte infiltration in primary CRC tumours, particularly CTLs and T_H1 cells with an interferon-γ (IFNγ)-dominant immune profile, positively correlated with relapse-free and overall survival[39–41]. Conversely, T_H17 cell infiltration and an interleukin-17 (IL-17)-dominant immune profile associated with poor outcomes[39]. A clinical translation of these findings was the establishment of a scoring system, called immunoscore, based on the abundance of two distinct lymphocyte populations ($CD8^+$ CTLs and CD45RO memory T cells) at the tumour centre and at its invasive margin[42]. Their quantification in early-stage CRCs is a validated prognostic marker, with 50% less risk of tumour relapse for those tumours with high immunoscores versus those with low immunoscores[43]. Subsequently, investigators have shown that the density of T cells decreased along with tumour progression, whereas the densities of B cells and T follicular helper (T_{FH}) cells increased from early-stage to more invasive CRC[41]. High B cell or T_{FH} infiltration in late-stage neoplasms correlated with prolonged disease-free survival[41]. Moreover, immune infiltration patterns and inflammatory cytokines have been linked to microbial dysbiosis and colon carcinogenesis. Tumours infiltrated with $CD4^+$ T cells that express the forkhead box P3 (FOXP3) transcription factor, which function as regulatory T (T_{reg}) cells and hinder effective immune responses against cancer cells, show significantly worse prognosis[44].

Recent studies have carried out a more comprehensive analysis of immune phenotypes in CRC (FIG. 3). It is well known that CRC tumours with defects in the DNA mismatch repair pathway, as indicated by MSI or hypermutation rates, display high infiltration with CTLs and activated T_H1 cells, which is counterbalanced with upregulated expression of multiple immune checkpoints[45–47]. Of note, CTL-associated antigen 4 (CTLA4), programmed cell death protein 1 (PD1), PD1 ligand 1 (PDL1) and indoleamine 2,3-dioxygenase 1 (IDO1) are highly upregulated across all hypermutated tumours with MSI[46–48]. The density of CTL infiltration into the tumour microenvironment positively correlates with the total number of frameshift mutations, suggesting that these genomic events can lead to the production of immunogenic neo-peptides, recognized by antigen-specific tumour infiltrating lymphocytes[49]. These tumours also have the highest expression of genes involved in T_H1 phenotype orientation (for example, *IFNγ* and *IL-15*), formation of tertiary lymphoid structures (for example, C-X-C motif chemokine ligand 13 (*CXCL13*)), chemokines attracting T cells (for example, *CXCL9*, *CXCL10* and *CXCL16*), and activation of the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) immune signalling pathway[28,41,46,47].

In addition to the highly immunogenic hypermutated MSI subtype of CRC (CMS1), the expression-profile analysis identified another cluster of tumours that display a different immune infiltration pattern. These tumours showed high expression of genes specific to T_{reg} cells, myeloid-derived suppressor cells (MDSCs), monocyte-derived cells and T_H17 cells, which are typically seen in the microenvironment of immune-tolerant malignancies[46,47]. This 'inflamed' immune subtype of CRC is characterized by marked upregulation of immunosuppressive factors, such as TGFβ and CXCL12 (REFS 46,47), and high expression of genes encoding chemokines that attract myeloid cells, including C-C motif chemokine ligand 2 (CCL2) and the related cytokines IL-23 and IL-17, which are known carcinogenic drivers in colitis-associated CRC[50,51]. The biological link between the inflamed immune CRC subtype and EMT is consistent with the finding that the stroma of CMS4 tumours is infiltrated not only with endothelial cells and CAFs but also with innate immune cells[28,47]. In addition, it suggests that the worse outcomes seen in the CMS4 mesenchymal population may be partially linked to a pro-metastatic immune evasive microenvironment. These results corroborate initial findings by Galon and others that an activated immune microenvironment in early-stage CRC is a strong determinant of the risk of distant dissemination[39,52] and that colitis-associated CRC is associated with an aggressive clinical behaviour[53].

Last, most CRC tumours, namely those that show MSS and are non-hypermutated and epithelial by gene expression (most CMS2 and CMS3 tumours), exhibit low immune and inflammatory signatures, lack tumour-infiltrating lymphocytes and immunoregulatory

Late-stage neoplasms
Localized tumours that have grown more deeply into nearby tissue or have spread to regional lymph nodes.

cytokines in the microenvironment and are typically PDL1-negative, which suggests that these tumours are poorly immunogenic[47,48]. WNT–β-catenin pathway signalling, which is activated in most epithelial tumours, correlates with T cell exclusion across solid tumours[54]. However, more detailed immune characterization of these tumours is needed. For instance, a recent study found higher levels of expression of T cell activation markers such as inducible T cell co-stimulator (ICOS) and immunostimulatory molecule OX40 in CMS3 primary carcinomas than in other subtypes[55].

Spatial and temporal molecular heterogeneity during CRC progression. All studies discussed above revealed the extensive inter-tumour heterogeneity of CRC at the genomic, epigenomic, transcriptomic and immune levels. Molecular heterogeneity has also been evaluated in tumours, both spatially and temporally. In CRC, data on intra-tumour heterogeneity are mostly limited to genomic studies that compare primary and metastatic sites. These studies found high concordance rates for mutations in the known driver oncogenes *KRAS*, *NRAS* and *BRAF*, which typically occur in a mutually exclusive manner[56,57]. Differences are higher for *PIK3CA* mutations and rarely mutated oncogenes, particularly after intervening chemotherapy, including *de novo TP53* mutations in liver metastasis[57]. In patients with acquired resistance to EGFR mAbs, discordances are more pronounced and the primary-metastasis genomic heterogeneity reflects a clonal selection process induced by treatment pressure. Mutations in the MAPK pathway, including *KRAS*, *NRAS* and *BRAF* mutations, emerge in a large proportion of tumour biopsy or circulating tumour DNA (ctDNA) samples from patients whose tumours were initially diagnosed as wild-type for *KRAS* pretreatment[58–64] (FIG. 4). In one-third of cases, multiple events coexist in the same sample[58,59,61,62], and the newly detected mutations seem to derive from minor pre-existing clones in the primary tumour lesion[61]. Repeated ctDNA analyses have shown that the percentage of *KRAS*-mutated alleles increases on anti-EGFR treatment and rapidly declines after drug withdrawal[61,62]. The number of *KRAS*-mutated clones was found to correlate inversely with the time since the last dose of EGFR mAbs[61,62] (that is, there are fewer clones as the time from the last dose increases) and to dynamically evolve on anti-EGFR therapy rechallenge (the number of clones increases)[62]. Finally, acquired *EGFR* mutations that affect the extracellular domain of the protein and thereby disrupt cetuximab binding have been recurrently identified in cetuximab-resistant CRC samples[58,59,61,62,65,66]. Interestingly, these *EGFR* mutations have not been identified in matched pre-treatment primary lesions, indicating that they are not implicated in innate resistance to anti-EGFR therapy, but frequently coexist with *KRAS* mutations[58,67]. Whether *EGFR* mutations are driver alterations or represent passenger genomic events emerging after long-term anti-EGFR treatment pressure needs further investigation.

In terms of copy number alterations and gene expression patterns, studies have shown striking similarities between primary tumours and matched metastases both at the genome-wide and gene-specific levels[68–71]. However, the diversity of profiling technologies, different patient characteristics and small sample size of most cohorts limit conclusive statements, especially because intervening treatment was rarely taken into account in these studies. For example, in the setting of anti-EGFR therapy, *MET* (also known as *HGFR*) and *ERBB2* amplifications are enriched in patient samples obtained at the time of progression compared with primary tumour samples, paralleling MAPK pathway mutations[64,72–75] (FIG. 4). In addition, inter-metastatic heterogeneity at the copy number level is higher in patients with metachronous disease who were previously exposed to chemotherapy than in chemo-naive patients[76]. Nevertheless, the lack of recurring acquired genomic and transcriptomic alterations in metastases compared with primary tumours reinforces the idea that the biological processes that drive metastases are defined early in the carcinogenesis process. Notably, primary-metastasis heterogeneity in CRC microenvironment markers has not been investigated in detail, but initial reports describe relevant changes in immune-cell infiltration patterns, such as fewer $CD8^+$ T cells and higher average frequencies of $CD68^+$ macrophages in metastases compared with primary tumours, both in the tumour centre and in the invasive margin[55].

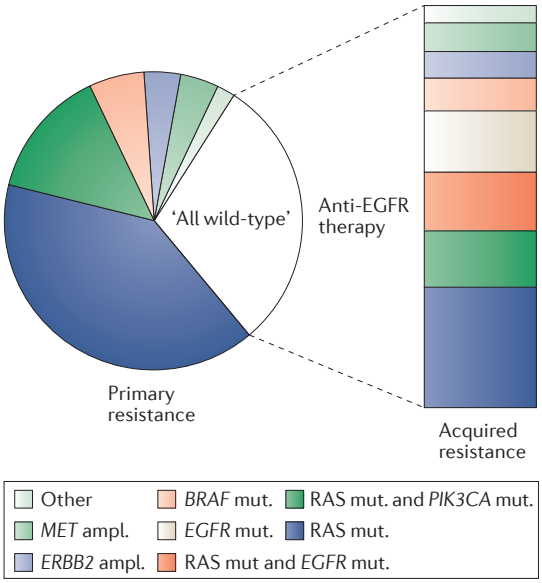

Figure 4 | **Genomic landscape before and after anti-EGFR therapy in advanced colorectal cancer.** The population of patients with no MAPK-pathway genomic alterations before treatment ('all wild-type') is more likely to respond to epidermal growth factor receptor (EGFR) monoclonal antibodies (mAbs). The high overlap in primary (left) and acquired (right) resistance mechanisms reinforces that clonal selection is a major determinant of the clinical outcome. Only *EGFR* mutations have not been identified in pre-treatment lesions. In a substantial proportion of the samples, resistance is polyclonal, with co-occurring RAS mutations and *EGFR* or PI3K catalytic subunit-α (*PIK3CA*) mutations. ampl., amplification; mut., mutation.

Rechallenge
Reintroduction of the same therapy after a drug holiday following disease progression during therapy.

Metachronous disease
Tumours that became metastatic after the diagnosis and treatment of localized disease (usually later than 6 months).

Selective sweeps
These occur when a rare or previously non-existing allele that increases the fitness of the cell — relative to other clonal populations — expands rapidly in frequency as a result of natural selection.

Underpowered
A study with low statistical power of detecting a true effect of practical importance.

Recently, investigators have assessed the spatial distribution of intra-tumour heterogeneity in CRC to infer the mutational timeline and the dynamics of tumour growth of established tumours[77]. Through genomic profiling of multiple individual glands from different CRC tumours, they found that subclonal expansions and selective sweeps are infrequent after progression to a late-stage neoplasm. As such, they proposed the 'Big Bang' model of CRC carcinogenesis, whereby the timing of a mutation's emergence determines whether it is present in all clones and remains pervasive during tumour growth. According to this model, CRC tumours grow predominantly as a single expansion populated by numerous intermixed clonal and subclonal alterations, with later genomic alterations localized in progressively smaller tumour subpopulations. Despite remaining non-dominant, subclonal events cooperate with other genomic alterations to sustain CRC progression. This observation is in line with the 'omics' studies described above showing major similarities between matched primary and metastatic samples on mutational, copy number and gene expression profiles. It is also consistent with reports that indicate that minor *KRAS*-mutated cell subpopulations that are intrinsically resistant to EGFR mAbs can contribute to poor treatment response and clonally expand, driving tumour growth after exposure to targeted agents[61]. Furthermore, this study highlights how an in-depth analysis of the spatial (multi-region) genomic heterogeneity in primary CRC can advance biology understanding[77], and opens the door to similar investigations in metastatic samples, with potential implications for precision medicine.

Shifting precision medicine paradigms

In past decades, most registered trials with targeted agents in CRC had no pre-planned biomarker analysis other than for exploratory investigations, did not stratify patients by biomarker-defined subgroups and were underpowered for such analyses. But important advances came from retrospective correlative analyses of clinical trials, including the link between *KRAS* exon 2 mutations and innate resistance to anti-EGFR therapy. This biomarker stratification model marked the first paradigm for precision medicine in CRC, the single-alteration perspective: 'one gene, one drug' (*KRAS* exon 2 mutations, avoidance of EGFR mAbs)[5]. However, most patients with *KRAS* wild-type CRC did not respond to cetuximab or panitumumab, suggesting that additional resistance mechanisms were still in place. After advances in drug design, the one gene, one drug paradigm was applied to the study of potentially positive predictive markers in CRC. However, the disappointing efficacy results with single-agent BRAF inhibitors in advanced-stage CRC with $BRAF^{V600E}$ mutations[78] or with MEK inhibitors in *KRAS*-mutated disease[79] indicated that the single-alteration perspective for targeted therapies in CRC had substantial limitations.

The introduction of next-generation sequencing to clinical trial samples and molecular pre-screening programmes (including ctDNA analyses) together with major advances in preclinical models (including patient-derived xenografts (PDXs) and organoids) meant that investigators were able to characterize patterns of co-occurring driver events, identify novel, rare, targetable alterations with potentially higher oncogenic dependency, understand the dynamics of target inhibition to design more rational drug combinations and recognize that temporal heterogeneity and clonal selection can explain resistance to matched targeted agents in CRC. These developments marked the emergence of the 'multi-gene, multi-drug' paradigm of precision medicine in CRC. With EGFR mAbs, for example, studies have shown that any benefit from these agents is restricted to patients with tumours that are wild type for all *KRAS* and *NRAS* loci (multi-gene), with an accompanying improvement in the cost–benefit ratio and drug efficiency when added to standard chemotherapy[5]. Combined inhibition of BRAF, MEK and EGFR (multi-drug) in $BRAF^{V600E}$-mutated CRC is another encouraging example, as it has led to unprecedented response rates in early clinical trials[80]. Emerging positive predictive markers for treatment selection in advanced-stage CRC are described in BOX 1 and TABLE 1. Unfortunately, for drugs that have multiple targets and are less well characterized in terms of the downstream pathways they influence — such as the multi-kinase inhibitor regorafenib, which affects VEGF receptor 1 (VEGFR1), VEGFR2, VEGFR3, RET, KIT, fibroblast growth factor receptor 1 (FGFR1), FGFR2 and platelet-derived growth factor receptor-α (PDGFRA), among others — biomarker discovery has proved more problematic. With the identification and characterization of transcriptomic and immune CRC subtypes, we have now a unique opportunity to revisit the best combination of biomarkers using a 'multi-molecular' framework that may help to predict response or resistance to anticancer therapies (FIG. 5).

Current multi-gene, multi-drug paradigm: EGFR mAb therapy as a framework. The study of innate and acquired resistance mechanisms to EGFR mAbs has revolutionized our knowledge on CRC biology and the dynamics of tumour progression. Nearly 70% of CRC samples have heterogeneous alterations in genes involved in EGFR signalling that confer resistance to cetuximab and panitumumab[5]. Indeed, gene-expression signatures that reflect downstream MAPK pathway activation predict the efficacy of anti-EGFR therapy better than *KRAS* mutations alone[81,82]. When considering the combination of *KRAS* and *NRAS* variants of exons 2, 3 and 4, the negative predictive value for response to EGFR blockade is so robust that they represent the primary model for disease subtyping and patient stratification in CRC: RAS wild-type versus RAS mutated[83,84]. This stratification has now been adopted in all treatment guidelines and represents a key set of biomarkers for standard-of-care management.

It was next discovered that close to 20% of *KRAS* wild-type tumours have a minor *KRAS*- or *NRAS*-mutated clone in the tumour when re-tested with a

Box 1 | **Emerging positive predictive markers for treatment selection**

BRAF^{V600E} *mutations*

In early trials that included patients with *BRAF*^{V600E}-mutated metastatic colorectal cancer (CRC), the combination of BRAF and MEK inhibitors has shown markedly lower clinical benefit than is observed in melanoma[142], possibly because the degree of MAPK pathway inhibition achieved with this combination therapy in CRC is suboptimal[142]. Indeed, the genomic alterations that result from acquired resistance to these agents in CRC all converge on MAPK reactivation[143,144]. Preclinical studies suggest that this pharmacodynamic failure may be partially driven by epidermal growth factor receptor (EGFR) activation as a feedback mechanism occurring in CRC but not melanomas, in which PI3K mediates sustained MAPK signalling[145,146]. Initial results of clinical trials evaluating combinations of EGFR monoclonal antibodies (mAbs), BRAF inhibitors and a PI3Kα inhibitor or a MEK inhibitor indicate that triple therapies may be more effective than other combination approaches in terms of response and progression-free survival[80,147].

ERBB2 *amplifications*

Patient selection has proved critical in the development of HER2 (encoded by *ERBB2*)-targeted agents in CRC. An early effort to recruit patients with *ERBB2*-amplified CRC for treatment with the HER2 mAb trastuzumab in combination with the standard-of-care chemotherapy irinotecan was halted owing to the low prevalence of the alteration, despite promising antitumour activity in the biomarker-positive population[148]. More recently, with a larger pre-screening effort in a heavily pre-treated *KRAS* wild-type subgroup of patients with advanced-stage CRC, clinical trial recruitment was successful and substantial clinical activity was seen with a dual HER2-targeted regimen, trastuzumab in combination with the tyrosine kinase inhibitor lapatinib[75].

ALK *and* NTRK1 *fusions*

Transcriptional outlier analysis identified RAS and *BRAF* wild-type CRC cells that are resistant to EGFR blockade and are functionally and pharmacologically 'addicted' to other kinase genes, including anaplastic lymphoma kinase (*ALK*), neurotrophic receptor tyrosine kinase 1 (*NTRK1*), *NTRK2*, *NTRK3*, fibroblast growth factor receptor 2 (*FGFR2*) and *RET*[149]. Indeed, rare samples from patients with CRC who have exceptionally high *ALK* and *NTRK1* expression levels were found to harbour genomic rearrangements involving these genes, which render tumours responsive to kinase inhibitors according to preclinical models and emerging clinical reports[18,150].

RNF43 *mutations*, ZNRF3 *mutations and* RSPO *fusions*

Organoid culture models identified genomic alterations associated with extremely high sensitivity to targeted agents. Cancers that are WNT ligand dependent owing to upstream alterations in the pathway (such as mutations in the ubiquitin ligase ring finger protein 43 (*RNF43*) or its homologue zinc and ring finger 3 (*ZNRF3*), and fusions in R-spondin (*RSPO*)) are predicted to be responsive to inhibitors of WNT secretion (porcupine)[151,152], and early signs of efficacy have been observed in patients with *RNF43*-mutated CRC[153]. Furthermore, the administration of RSPO3-neutralizing mAbs to xenografts derived from patients with *RSPO3*-fusion-positive CRC induced tumour growth inhibition that persisted after treatment cessation. This inhibitive effect was linked to tumour differentiation and effects on stem-cell compartment genes[154].

MSI and* POLE *mutations

The link between high somatic mutation loads and immune activation in tumours with microsatellite instability (MSI) has translated into encouraging efficacy of single-agent programmed cell death protein 1 (PD1) checkpoint inhibitors in mismatch repair-deficient CRC, in contrast to lack of efficacy in an unselected mismatch-repair-proficient population[155,156]. Hypermutation rates are not exclusively seen in tumours with MSI. DNA polymerase-ε (*POLE*)-mutated tumours that show microsatellite stability, for example, also harbour high neoantigen loads and tumour-infiltrating lymphocytes in their microenvironment[157]. To avoid immune attack, multiple immune checkpoint receptors are hijacked by *POLE*-mutated tumour cells[46,48], a finding that supports the investigation of PD1 mAb therapy.

highly sensitive sequencing technology[85,86]. The presence of a low fraction (as low as 1%) of mutated cells in primary tumours may provide a reservoir for acquired resistance to EGFR mAbs. Indeed, measurements of mutant allele fractions in samples of primary tumours correlate with the effects of anti-EGFR therapy in the advanced setting[83,87]. This finding not only corroborates the known concordance of mutation events in primary and metastatic samples for RAS genes (before treatment with EGFR targeted agents) but also suggests that relapsed lesions are likely to retain the genomic structure of early colorectal tumours[88].

However, innate resistance of tumours to EGFR blockade is also influenced by additional pathway alterations, including mutations in *BRAF*, *MEK1*, *ERBB2*, *FGFR1* and *PDGFRA*[66,89]. The rarity of these events limits the assessment of their clinical value as negative predictive biomarkers, but preclinical models showed durable responses with a targeted approach that inhibits these resistance mechanisms in combination with anti-EGFR therapy[66,89]. In the setting of emergent *ERBB2* mutations, for example, HER2-targeted therapy in combination with EGFR mAbs produced major tumour regressions in PDXs[89].

Tumours that are wild type for *KRAS*, *NRAS*, *BRAF* and *PIK3CA* (quadruple negative), which represent up to 30% of cases, are more likely to respond to anti-EGFR therapy[90]. This population is being accrued in clinical trials that assess rechallenge with EGFR mAbs, based on the hypothesis that pre-existing sensitive subclones emerge after treatment breaks[61,62]. Quadruple wild-type tumours are particularly sensitive to dual EGFR targeting, including tyrosine kinase inhibitors (TKIs) in combination with EGFR mAbs, both of which inhibit EGFR through different mechanisms[91]. Another strategy is the administration of mixtures of mAbs that target non-overlapping epitopes of the EGFR extracellular domain, such as Sym004 (REF. 74) and MM-151 (REF. 92), which are designed to induce a higher degree of receptor internalization and degradation. Encouraging preliminary efficacy results were seen with Sym004 in patients diagnosed with acquired resistance to EGFR mAbs[74]. Furthermore, dual EGFR targeting is a promising strategy in patients with tumours showing *EGFR* ectodomain mutations[66,74]. A subset of these mutations acquired under cetuximab treatment may be permissive for interaction with panitumumab, as panitumumab binds to a distinct epitope of the molecule. Indeed, case reports of transient responses to panitumumab and Sym004 were documented in the setting of acquired *EGFR*^{S492R} mutations[74,93].

In preclinical models, the extensive crosstalk among ERBB family receptors leads to upregulation of parallel pathways after EGFR blockade as a compensatory adaptive resistance mechanism[5]. However, clinical trials with unselected populations evaluating alternative agents or EGFR mAbs in combination with drugs targeting 'escape' signalling pathways — such as HER3 (REF. 94), insulin-like growth factor 1 receptor (IGF1R)[95,96] and MET[96,97] — had negative efficacy results. These failures may be related to the lack of a reliable biomarker for patient stratification or the insufficient potency of the targeted agent. Nevertheless, it is clear that non-genetic mechanisms also play a part in

Table 1 | **Emerging positive predictive biomarkers for treatment selection in advanced CRC**

Alteration	Prevalence in advanced CRC (%)	Agents	Clinical phase	Partial response (*n*/*n* (%))
BRAF^{V600E} mutations	5–8	BRAF TKI + MEK TKI	Phase II	5/43 (12)[142]
		BRAF TKI + MEK TKI + EGFR mAbs	Phase II	9/35 (26)[80]
		BRAF TKI + PI3K TKI + EGFR mAbs	Phase II	9/28 (32)[147]
ERBB2 amplification	5*	Anti-HER2 mAb + pan-ERBB TKI	Phase II	8/27 (30)[75]
NTRK1 fusion	<1	NTRK TKI	Phase I	Case report[150]
ALK fusion	<1	ALK TKI	Phase I	Case report[18]
RNF43 mutations	<5	Porcupine inhibitor	Phase I	Case report[153]
MSI	<5	PD1 mAbs	Phase II	4/10 (40)[155]
				9/33 (27)[156]

ALK, anaplastic lymphoma kinase; CRC, colorectal cancer; EGFR, epidermal growth factor receptor; mAb, monoclonal antibody; MSI, microsatellite instability; NTRK, neurotrophic receptor tyrosine kinase; PD1, programmed cell death protein 1; *RNF43*, ring finger protein 43; TKI, tyrosine kinase inhibitor. *Of patients with *KRAS* wild-type tumours.

resistance to anti-EGFR agents. Complete responses to cetuximab or panitumumab therapy are extremely rare, and only a fraction of cancer cells in tumour samples from patients with progressive disease carry activating MAPK pathway mutations, which suggests that wild-type cells can also survive the treatment[60,74]. Preclinical findings point to conservation of EGFR dependency in tumours that progress on anti-EGFR therapy; this is possibly related to adaptive ligand overexpression in the tumour microenvironment and paracrine interactions between *KRAS*-mutated and wild-type subclones[98–101]. In addition to sustained EGFR dependency, recent pathway-oriented genetic screens have revealed downstream activation of MAPK signalling as a bottleneck in CRC cells that escape EGFR blockade. In quadruple-negative CRC PDX samples, vertical (upstream and downstream) suppression of EGFR signalling with the combination of anti-EGFR and anti-MEK therapy prevents the development of acquired resistance[102]. Interestingly, this targeted combination was already tested in a constitutively *KRAS*-mutated CRC population with negative results, which were possibly related to insufficient target inhibition[103]. Early exposure of RAS wild-type tumours to EGFR and MEK inhibitors, when *KRAS* mutations may be found in minor subclones, is expected to increase the chances of complete pathway blockade.

Future 'multi-molecular' paradigm

Gene alterations in the context of transcriptomic subtypes. Even though CMS groups are enriched for key genomic markers such as *KRAS* and *BRAF* mutations, the transcriptional signatures allow further refinement of disease subclassification. For example, RAS wild-type tumours are considered to be a homogenous entity for therapeutic decisions in patients with advanced-stage CRC, despite being found across distinct CMS groups with profound biological differences, which are expected to translate into heterogeneous drug responses. Indirect evidence suggesting that gene-expression signals determine sensitivity to EGFR blockade comes from exploratory analyses of clinical trials that assessed the differential benefit of EGFR mAbs according to an anatomical classification: that is, proximal carcinomas (right colon, from caecum to transverse colon) versus distal carcinomas (left colon, from splenic flexure to rectum)[104,105]. Researchers have found significant interactions between tumour site location and clinical outcome with or without cetuximab in patients with *KRAS* wild-type advanced-stage CRC; survival benefit of the anti-EGFR therapy was restricted to patients diagnosed with a distal primary tumour[104,105]. This interaction may be partially explained by underlying biological differences between proximal and distal carcinomas at the transcriptomic level. Proximal tumours, which are enriched for CMS1 and CMS3 subtypes, show reduced expression of the EGFR ligands amphiregulin (AREG) and epiregulin (EREG); this reduced expression is linked to hypermethylation of the ligands' promoter regions[106]. It is also known that distal carcinomas, particularly of CMS2 phenotype, frequently overexpress EGFR ligands[28,106,107] and harbour amplifications of *EGFR* and *IRS2*, which are markers of cetuximab sensitivity[66,108]. But additional oncogene alterations that potentially drive resistance to EGFR mAbs in RAS wild-type patients are also enriched in the CMS2 population, including actionable *ERBB2* and *IGF2* copy number gains[109], making it the most appealing group to test combinations of pan-ERBB and IGF1R inhibitors. On the contrary, RAS wild-type tumours with a mesenchymal phenotype seem to be intrinsically resistant to anti-EGFR agents in preclinical models[26]. In fact, retrospective biomarker analyses of a patient cohort in the chemotherapy-refractory setting[22] and a randomized clinical trial in the chemo-naive setting[110] suggest no benefit of treatment with cetuximab in patients with mesenchymal-like tumours. Potential targets that may increase the efficacy of EGFR mAbs in the RAS wild-type CMS4 population include drivers of EMT and treatment resistance, such as MET[26] and integrins[111]. In a clinical report, combination therapy with cetuximab and a mAb anti-integrin-αv was particularly effective in patients whose tumours displayed high integrin-αvβ6 expression levels[112], which is typically seen in CMS4 mesenchymal samples[28].

Caecum
The first segment of the right colon, an intraperitoneal pouch that connects the ileum with the ascending colon.

Splenic flexure
The first segment of the left colon, a sharp bend under the spleen where the transverse colon joins the descending colon.

Adjuvant
Therapy applied after initial surgical treatment for cancer to avoid tumour relapse.

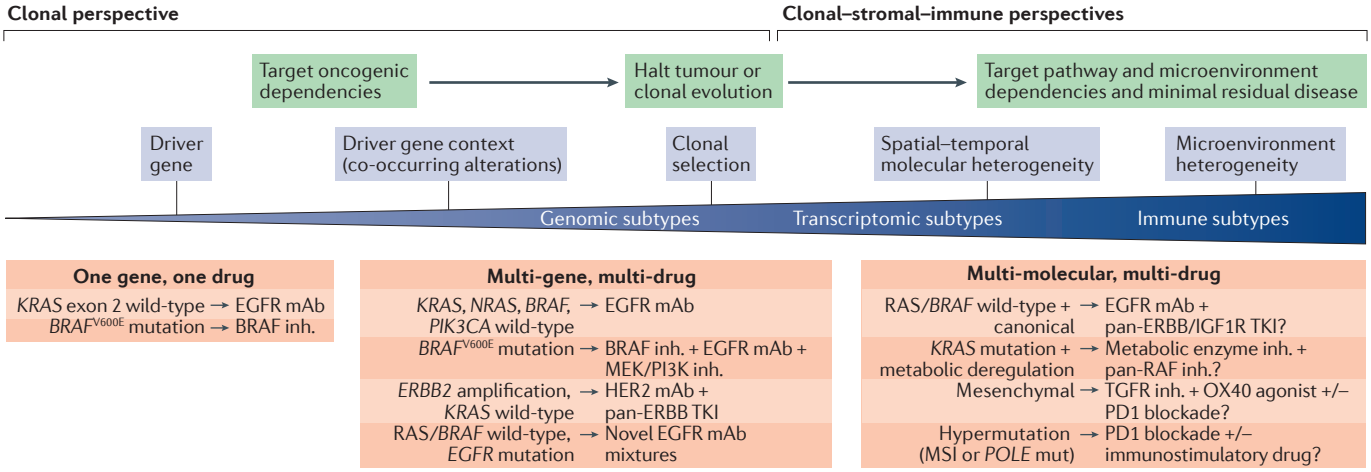

Figure 5 | **Evolution of precision medicine paradigms in colorectal cancer.** The shift from a clonal perspective for targeted therapies ('one-gene, one-drug' and 'multi-gene, multi-drug') to a clonal–stromal–immune perspective ('multi-molecular, multi-drug') reflects increased understanding of the biology of the disease and advances in biomarker–drug co-development. EGFR, epidermal growth factor receptor; IGF1R, insulin-like growth factor 1 receptor; inh., inhibitor; mAb, monoclonal antibody; MSI, microsatellite instability; PD1, programmed cell death protein 1; *PIK3CA*, PI3K catalytic subunit-α; *POLE*, DNA polymerase-ε; TGFR, transforming growth factor-β receptor; TKI, tyrosine kinase inhibitor.

Although therapy-optimization strategies in patients with RAS wild-type CRC are unlimited, targeted treatment of *KRAS*-mutated disease has proved extremely difficult and has not evolved in recent years. For instance, despite strong scientific rationale and preclinical data supporting the combination of MEK inhibitors and PI3K pathway inhibitors, no clinical activity was seen in CRC[113]. *KRAS*-mutated CRC is highly heterogeneous at the gene expression level, with unique metabolic dependencies in tumours with a CMS3-dominant phenotype. Context-specific molecular susceptibilities have been identified in *KRAS*-mutated lung carcinomas, such as deficits in nucleotide metabolism and lysosomal maturation[114–116]. Interestingly, the subgroup of *KRAS*-mutated lung carcinomas with coexisting amplifications of the *KRAS*-mutated allele is characterized by marked rewiring of glucose metabolism towards glutathione biosynthesis[36], mirroring the CMS3 metabolic adaptation seen in CRC. *KRAS*-mutated homozygous lung cancer cell lines are particularly sensitive to low glucose levels and glutathione synthesis inhibitors[36]. Remarkably, high-dose vitamin C has been shown to impair CRC tumour growth in mouse models by causing oxidative stress and glutathione depletion selectively in *KRAS*-mutated cells[117]. Novel inhibitors of metabolic enzymes, such as glutaminase[118] and fatty acid synthase (FASN)[119], are in early clinical development and should be revisited as targeted interventions in *KRAS*-mutated CRC tumours.

Pathway activation in the context of transcriptomic subtypes. The different CMS groups have unique pathway enrichment traits that may be selectively targeted in the clinic. Stratifying CRC cell line panels according to gene-expression classifiers and overlaying pharmacological response data for targeted therapies showed important differences in sensitivity across cell lines assigned to different subtypes[27]. For instance, an increased response of highly proliferative epithelial-like CRC cells to aurora kinase inhibitors was identified[27]. However, before clinical translation, a deeper understanding of the molecular processes that regulate dynamic changes in pathway activation and the mechanisms of action of targeted agents is needed. The identification of actionable targets in CMS4 mesenchymal tumours is of major interest, considering the higher chances of metastatic spread. There is strong evidence that stromal cells mediate resistance of CRC cancer cell lines to chemotherapies and targeted agents[120]. Indeed, retrospective analysis of a randomized clinical study showed poor prognosis and no benefit from adjuvant oxaliplatin-based chemotherapy in the subset of patients with stage III CRC whose tumours displayed a mesenchymal phenotype[121]. In preclinical models, the use of TGFβ signalling inhibitors to block the crosstalk between cancer cells and the microenvironment was shown to halt disease progression of stromal-enriched poor prognosis CRC tumours[122]. The combination of chemotherapy with a TGFβ receptor (TGFR) inhibitor has already moved to clinical trials in patients whose tumours test positive for a 'TGFβ activated' signature as part of the MoTriColor project, a large pan-European effort that is pioneering molecularly guided trials in metastatic CRC[123]. Similarly, signalling activation of UFO (a tyrosine-protein kinase receptor encoded by *AXL*) and NOTCH networks also triggers EMT in CRC and is associated with an aggressive tumour phenotype and resistance to targeted agents[124,125]. Indeed, both pathways are overactive in CMS4 mesenchymal CRC[28], thereby providing novel leads for pharmacological inhibition in this metastasis-prone subtype of the disease.

Immune activation in the context of transcriptomic subtypes. Integrating knowledge from immune and transcriptomic subtyping of CRC may guide novel immunotherapeutic strategies, particularly for the inflamed mesenchymal population (FIG. 3). The idea is to overcome multiple mechanisms that mediate immune tolerance to self-antigens and block the intense immunosuppressive response in the tumour microenvironment. The pro-tumorigenic functions of TGFβ are mediated not only through direct action on tumour cells but also through its effects on immune cells — inhibition of CTLs, T_H1 cells and NK cells, and expansion of T_{reg} cells, B cells and MDSCs. Therefore, for an immunotherapy to be successful in inflamed mesenchymal tumours, it is likely to require not only an agonist to boost effector CTL function but also inhibitors of T cell suppression. In mouse models of highly aggressive mesenchymal CRC tumours, a potential synergistic effect was observed with the combination of a TGFR inhibitor with a PD1 checkpoint inhibitor[126], or with an agonistic OX40 mAb[127], which enhances effector function and survival of activated T cells. The positive treatment outcome was associated with an expansion of tumour-infiltrating effector CTLs and T_H1 cells, enhanced antitumour T cell immunity[126,127], and a high tumour-specific IFNγ response[127]. TGFR inhibitors also significantly improved the efficacy of subsequent radiotherapy in a preclinical model of rectal cancer[128]. The researchers were able to show that this effect was mainly dependent on adaptive immunity and related to an improved immune microenvironment rather than changes in radiosensitivity, EMT or angiogenesis markers. Alternative immunotherapeutic approaches to be explored in inflamed mesenchymal tumours include pharmacological elimination of MDSCs or blockade of related immunosuppressive chemokine signalling circuits and pathways, as demonstrated in other malignancies with an immune-evasive microenvironment[129].

For poorly immunogenic or immune-ignorant CRC tumours, complementary therapeutic approaches to checkpoint inhibitors are also needed. These include cancer vaccines with dendritic cells to stimulate tumour infiltration with antigen-specific CTLs[130], or alternative agents that can increase expression of T cell chemokines and enhance T cell infiltration in a non-antigen-specific way, such as histone deacetylase (HDAC) inhibitors[131]. Despite negative results with checkpoint inhibitors as monotherapies in patients with tumours that show MSS, multiple trials are investigating the value of combined administration of standard chemotherapies known to induce immunogenic death of CRC cells, such as oxaliplatin[132], and anti-angiogenic agents that may neutralize vascular barriers preventing T cell homing in the microenvironment, including bevacizumab[133]. Importantly, it is still unclear to what extent chemotherapies and targeted agents affect the tumour microenvironment. In mouse models, MEK inhibition induced intratumoural T cell accumulation and major histocompatibility complex (MHC) class I upregulation, and synergized with immune checkpoint inhibition to promote durable tumour regression[134]. Indeed, preliminary data from a clinical trial assessing the combination of a MEK inhibitor with anti-PDL1 agent showed early signs of efficacy in patients with MSS non-hypermutated CRC[135]. Another strategy under investigation is the combination of immune modulators and anti-EGFR therapy in a RAS wild-type population, reflecting the notion that the immune system substantially contributes to the therapeutic effects of mAbs[136].

Conclusions

All of the accumulated knowledge on CRC biology needs to be considered when shaping the future clinical development of targeted agents. The identification of molecularly homogeneous subsets of CRC — and the characterization of driver events in these tumours — will certainly advance drug development strategies. It is time to integrate novel technologies for biomarker discovery and advance to a 'multi-molecular, multi-drug' paradigm for precision medicine, whereby the evolution of clonal cancer cell events, expression of cancer pathway components and interactions with the tumour microenvironment are taken into consideration. Clinical trial design is also evolving to accommodate the new molecular paradigm. In this context, adaptive frameworks for patient accrual and drug selection are crucial for a successful proof-of-concept, allowing for drug rechallenge and flexible patient stratification algorithms to target acquired resistance mechanisms[137]. Here, we discuss the prospects of this paradigm (TABLE 2).

First, subclassification per se, even when built on allegedly relevant features of tumour, stromal and immune cells, may still not be predictive of differential drug responses. This can be due to the drugs themselves, which can have promiscuous mechanisms of action that may not track well with single pathway descriptors, or to our inability to properly define pathway engagement or crosstalk using static 'omics' data. Moreover, we still need to evaluate intra-patient molecular heterogeneity between primary samples and different metastatic lesions in larger CRC cohorts. Dynamic changes in genomic, transcriptomic and immune activation profiles have to be assessed in the light of intervening chemotherapy and administration of targeted agents.

Second, the insights into drug matches for specific gene expression or immune CRC subtypes discussed here are based on preclinical hypotheses or retrospective exploratory analyses of clinical cohorts with associated shortcomings. An understanding of the mechanisms that underlie therapeutic sensitivity or resistance requires the development of robust biomarker discovery programmes that use systems biology approaches with orthogonal interrogation of data sets. We think that novel contexts of vulnerability are likely to be identified, leading to drug-repurposing strategies. In addition, any emerging biomarker has to be put into context with driver gene mutations, MSI status, CMS and immune CRC classifications. We strongly support the following ideas: investigating

Table 2 | **Prospects for clinical translation of molecular tests in CRC**

Molecular testing	Objective	Timing	Examples — clinical translation
Next-generation sequencing (mutations, copy number alterations, fusions) and MSI	Target identification for matched therapies	At diagnosis of advanced CRC or progression on standard therapies	*ERBB2* amplification for HER2-targeted therapy, MSI for immune checkpoint inhibitor
ctDNA analysis	Detection of acquired resistance mechanisms and inter-metastatic genomic heterogeneity	At baseline and progression on treatment with targeted therapies	*EGFR* mutations during anti-EGFR therapy for novel EGFR mAb mixtures
	Prediction of radiological tumour progression	During standard therapy	Early change in therapy to alternative rescue regimen[158]
	Detection of minimal residual disease	Post-operative in stage II disease	Personalized adjuvant therapy[159]
Gene-expression classifiers (for example, CMS and supervised predictive signatures)	Subtype identification for matched therapies	Early or advanced-stage CRC (CMS classifier optimized for primary tissue)	Personalized adjuvant therapy for high-risk mesenchymal tumours, target validation in advanced-stage CRC
Immune markers (for example, proteomics in tumour microenvironment, immunophenotype and neoantigen detection)	Identify response and resistance biomarkers	At baseline, on treatment and progression to immunotherapies	Combination of immunotherapies for advanced-stage CRC with MSS

CMS, consensus molecular subtype; CRC, colorectal cancer; ctDNA, circulating tumour DNA; EGF, epidermal growth factor; EGFR, EGF receptor; mAb, monoclonal antibody; MSI, microsatellite instability; MSS, microsatellite stability.

the value of gene classifications in different preclinical models with drug sensitivity data; correlating the response patterns of approved and experimental targeted agents with the CMS classification in existing large clinical trials; adapting the design of future trials, such as adding stratification features or increasing their power to allow these retrospective correlative analyses to be carried out; and designing prospective clinical trials in advanced-stage CRC that incorporate new biomarkers with drug repositioning and/or novel matched targeted therapies. With regard to CMS classification in a research setting, the available models need to be optimized for subtype prediction on tissues in which microenvironment content is different from primary colorectal tumours, such as metastatic lesions and PDXs[29]. Different academic groups are working on a practical and robust CMS classifier that works on formalin-fixed, paraffin-embedded primary CRC tissues, based on either gene expression or immunohistochemistry[110]. The stromal dependence in CMS classification also suggests that standardized procedures should be used in tissue sampling for molecular classifiers to be clinically translated in prospective trials[30].

Third, rational combinations of targeted therapies will be required to achieve meaningful effects in different subtypes, with overlapping toxic effects that may further complicate the biomarker–drug co-development path. In the setting of actionable genomic alterations detected in tumours samples or ctDNA, for example, an additional layer of complexity is the rarity of most events (such as *ERBB2* and *MEK1* mutations) and the need to adapt therapies accordingly upon progression. Rather than targeting emergent subclones, an alternative approach is to develop treatment strategies that can halt tumour evolution. This could be achieved with intermittent administration of targeted therapies, vertical inhibition of convergent pathway alterations to delay the emergence of resistance clones, or the drugging of 'truncal' genomic events such as mutations in the WNT–β-catenin and MAPK pathways. Even in tumours that regress after treatment with targeted therapies, such as EGFR blockade, subsets of drug-tolerant cells often remain, fostering tumour relapse. To improve clinical benefit further, it is crucial to understand how residual disease is sustained and how it can be therapeutically tackled. The potential effect of immunotherapies on drug-tolerant clones and cells that lack targetable genetic alterations is being extensively investigated. To mount a tumour-rejection adaptive response and broaden the clinical activity of immuno-oncology drugs in non-MSI CRC subtypes, multiple combinations with checkpoint inhibitors may be explored, which represents a complex prioritization task. These combinations include T cell co-stimulatory agents, small-molecule immunomodulators and inhibitors of immunosuppression, chemokines, vaccination, targeted agents, cytotoxic drugs and radiation therapy.

Last, advances in patient stratification and drug development strategies have to be rapidly translated from the metastatic to the adjuvant setting. The most recent adjuvant clinical trials have not shown any value for adding targeted agents to standard chemotherapies in unselected stage III colon cancer[138–141]. A recent study uncovered the major impact of the host adaptive immune response on metastatic seeding in CRC[52]. Pathways that coordinate the creation of an immunosuppressive microenvironment and stromal invasiveness are strongly enriched in the CMS4 mesenchymal CRC population. We believe that a better understanding of the drivers of this pro-metastatic state will guide drug selection in future biomarker-driven adjuvant clinical trials and, hopefully, increase cure rates and survival in CRC.

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Competing interests statement

The authors declare no competing interests.