Cancer Treatment Reviews 73 (2019) 31–40

Contents lists available at ScienceDirect

Cancer Treatment Reviews

journal homepage: www.elsevier.com/locate/ctrv

Tumour Review

Integrative molecular analysis of colorectal cancer and gastric cancer: What have we learnt?

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ARTICLE INFO

Keywords:
Gene expression profiles
Molecular subclassification
Biomarkers
Gastrointestinal cancers

ABSTRACT

Gastrointestinal (GI) malignancies comprise a diverse group of cancers with varying aetiology, clinical course, management and prognosis. Advances over the last decade in molecular diagnostics in colorectal cancer (CRC) have helped to improve our understanding of the underlying complex mechanisms in the development and progression of this highly heterogenous disease. Large scale integrative analysis has identified molecularly distinct subgroups of CRC with differing clinical behaviour. It was hoped that these discoveries would fuel the development of novel drug targets and new treatments to shift the management of advanced CRC from an empirical strategy to a biomarker driven approach based on underlying molecular characteristics. However, biomarkers in current clinical practice remain limited in CRC. Gastric cancer (GC) has also been slow to benefit from biomarker discovery and development and the successful utilisation of targeted therapies, with the exception of trastuzumab in HER2 positive cancers. More recently, molecular analysis of GC has also identified distinct subgroups within these cancers with differing behaviour and therapeutic targets. In addition, our deeper understanding of the underlying molecular biology of GI cancers has led to the consideration of alterations above and beyond gene mutations. The clonal, stromal and immune characteristics of GI malignancies are increasingly recognised as important in therapeutic targeting. The challenge remains to apply the data generated through molecular exploration into clinical practice in order to provide personalised treatment to each individual patient.

Introduction

Gastrointestinal (GI) cancers collectively comprise a major healthcare burden globally with significant associated morbidity and mortality rates. Colorectal cancer (CRC) is the third most common cancer and fourth most common cause of cancer-related death worldwide [1]. Oesophageal and gastric cancers comprise the sixth and third most common cause of cancer-related death worldwide respectively [2], with significant geographical variation. The highest incidence and mortality rates for gastric cancer (GC) are in East Asia, reflecting the particular epidemiological factors in this region of the world. In addition, marked disparities are seen in GC outcomes between Eastern and Western populations and there is increasing evidence to suggest that underlying tumour biology, in addition to environmental and surgical factors, significantly contributes to these disparities [3]. This geographical variation in outcomes is not generally seen in CRC.

CRC and GC have varying clinical course and outcomes with differing management strategies, but the mainstay of treatment for both has historically involved surgery and chemotherapy. The majority of patients presenting with locally advanced (unresectable) or metastatic disease are managed with systemic chemotherapy. Advances in molecular diagnostics and biomarker discovery have led to the 'one gene, one drug' [4] application of targeted therapies to improve clinical outcome, such as trastuzumab in human epidermal growth factor receptor 2 (*HER-2)* overexpressed gastric cancer and cetuximab/panitumumab in *KRAS* wild-type colon cancer. More recently, integrated analysis within large international collaborations has led to the identification of distinct molecular subgroups within these tumours [5]. Translating these findings into optimal clinical practice however remains challenging and requires biomarker-drug co-development.

Here we outline the advances made in our understanding of the molecular landscape of CRC from retrospective biomarker discovery and validation through to more recent molecular subclassification via large scale data sharing and analytics. We also discuss how molecular characterisation has increased our understanding of the biology of GC which, thus far, has also been slow to benefit from biomarker discovery,

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https://doi.org/10.1016/j.ctrv.2018.12.004
Received 7 August 2018; Received in revised form 18 December 2018; Accepted 20 December 2018

molecular exploration and rationalised therapies. We explore how the molecular subgrouping of these cancers has or has not led to the discovery of predictive and prognostic biomarkers and whether their clinical utility has been exploited. Ultimately, this will be with the aim of streamlining biomarker and drug development in order to recruit patients to innovative clinical trials for selected therapies to which they are more likely to respond based on the underlying molecular characteristics of their individual cancers: true 'personalised medicine'.

Early classification of CRC and GC

CRC was one of the earliest molecularly characterised solid tumours. The sequence of CRC development in a step wise manner from adenoma formation to carcinoma through accumulation of genetic and epigenetic events was originally described by Vogelstein et al. [6]. This model provided insight into how driver alterations in the main oncogenes (*KRAS, NRAS, BRAF, PI3K*) and tumour suppressor genes (*APC, TP53, PTEN*) were implicated in the biology of CRC. The accumulation of these genetic mutations leads to carcinogenesis through deregulation of key pathways involved in cell proliferation, differentiation and apoptosis including Wnt/β-catenin, TGF-β, MAPK and PI3K signalling [7], with alterations in these pathways being almost universal in CRC.

The majority of sporadic CRC cases (85%) exhibit chromosomal instability (CIN) and display multiple structural chromosomal changes such as translocations, allelic losses, amplifications and mutations of *APC* and *KRAS* in the adenoma-carcinoma sequence [6]. This results in a highly heterogenous group with differing clinicopathological and prognostic characteristics. The remaining 15% of CRCs demonstrate microsatellite instability (MSI) through changes in the number of repeats or length of microsatellites [8]. MSI is caused by defective DNA mismatch repair (MMR) mechanisms which arise in one of two manners: through germline mutational inactivation of genes encoding MMR proteins, namely *MLH1, MSH2, PMS2 and MSH6* (in the Lynch familial syndrome) or sporadically by epigenetic silencing of the *MLH1* gene by promotor hypermethylation [9,10]. Epigenomic studies have shown that MSI tumours have a high CpG island methylator phenotype (CIMP-H). Abberant methylation of CpG rich gene promotor regions leads to silencing of expression of critical genes such as *MLH1*, thereby leading to the development of CRC [11].

Historically, GC has been classified using histopathological features. The majority of gastric cancers are adenocarcinomas and the Lauren classification system [12] divides them into two subtypes: diffuse (undifferentiated) or intestinal (well differentiated). The former is known to be linked with familial genetic disorders, such as Hereditary Diffuse Gastric Cancer and Lynch syndrome, whereas the latter is more typically associated with *H. Pylori* infection. The later developed World Health Organisation (WHO) system [13] describes a four group classification system, subdividing gastric cancers into papillary, tubular, mucinous and poorly cohesive. Whilst these defined subtypes demonstrate the heterogeneity of GC and allow differing epidemiological associations to be appreciated, they play no clear role in defining prognosis, predicting response or indeed guiding treatment decisions for individual patients.

History of biomarker discovery in CRC and GC

Biomarker discovery in CRC has been typified by the identification of negative predictive markers to the so-called targeted therapies that came into clinical practice over ten years ago. Early clinical trials of these targeted agents in CRC lacked any pre-planned biomarker analysis although patients were selected by EGFR expression detectable by immunohistochemistry (IHC) [14,15]. No correlation was found between EGFR expression and response however. A major breakthrough in biomarker discovery to anti-EGFR therapy arose from our initial understanding of clinical behaviour (through responders vs non-responders) and the retrospective analyses of large prospective clinical trial datasets.

The subsequently identified association between *KRAS* exon 2 mutations and resistance to anti-EGFR therapy marked the first era of precision medicine in CRC [16,17]. However, it became apparent that other resistance mechanisms were still in place when the majority of *KRAS* wild type patients failed to respond to anti-EGFR therapies [18]. This led to more extensive analysis of the *KRAS* gene beyond exon 2 as well as analysis of the *NRAS* gene. Although not statistically powered for such, secondary analysis of key studies involving EGFR inhibitors in CRC (PRIME, CRYSTAL, OPUS, FIRE-3, PEAK [19–23]) revealed no benefit of anti-EGFR antibodies in patients who have tumours harbouring these further mutations.

To add further to the *KRAS* story, evidence accumulated to suggest that not all *KRAS* exon 2 mutations inferred resistance to anti-EGFR therapy. Building on preclinical data which suggested *KRAS* codon 13 mutated tumours were in fact sensitive to cetuximab, a pooled analysis of 579 chemorefractory patients treated with cetuximab identified a significantly higher response rate (RR) and progression-free survival (PFS) in patients with codon 13 mutations compared to those harbouring other *KRAS* mutations [24]. An attempt to validate these findings was made in a pooled analysis of untreated metastatic CRC patients from the randomised CRYSTAL and OPUS datasets [25]. Patients with *KRAS* codon 13 mutations (G13D) fared worse in the control arm but better in the cetuximab-containing arm in both studies, leading to a high benefit from treatment effect for this subgroup. Patients with other *KRAS* mutations continued to exhibit worse outcomes when receiving cetuximab alongside chemotherapy. However, a prospective randomised phase II study by Segelov and colleagues [26] revealed no significant improvement in disease control at six months with either cetuximab monotherapy or cetuximab plus irinotecan in G13D mutated CRC patients. There were no responses seen with cetuximab monotherapy. This study highlights the necessity to prospectively evaluate hypotheses generated from retrospective analysis of datasets, particularly for rare mutations where responses in only a few patients can skew results. The full biomarker implications of different *KRAS* mutations are yet to be fully explored but, at the current time, extended *RAS* testing (*KRAS* exons 2 and 3 and *NRAS* exons 2,3 and 4) is seen as standard of care prior to considering anti-EGFR therapy in advanced CRC [27].

Unlike other cancers such as melanoma and GIST, disappointing results have been seen with direct targeting of other single predictive markers in CRC such as monotherapy with *BRAF* inhibitors in advanced $BRAF^{V600E}$ mutated disease [28]. $BRAF^{V600E}$ mutation in early and advanced CRC represents a poor prognostic biomarker [29,30] and is present in approximately 8% of cases. There is evidence to suggest it also confers resistance to EGFR-directed therapies [31]. Upon BRAF inhibition, CRC cells rely on feedback activation of EGFR via MAPK signalling and based on this, early clinical trials evaluating combined blockade of *BRAF*, *EGFR* and *MEK* in $BRAF^{V600E}$ mutated advanced CRC have shown promising response rates [32,33]. Also, the addition of BRAF inhibitor vemurafenib to cetuximab and irinotecan significantly improves progression-free survival and disease control rates in these patients too [34], highlighting the potential of a multi-drug approach in overcoming resistance mechanisms to improve efficacy.

In GC, the discovery of a subgroup of patients (up to 20%) demonstrating overexpression or amplification of *HER-2* [35] was the first clue that gastric cancer held molecular secrets that may hold the key to improving treatment outcomes. The subsequent successful therapeutic use of trastuzumab in combination with chemotherapy [36] and validation of *HER-2* as a predictive biomarker is proof of principle but the impact is still modest suggesting that multiple targets are likely to be needed for a major impact. Another therapeutic gain has been achieved with the vascular endothelial growth factor receptor 2 (*VEGFR-2*) antagonist, ramucirumab. The role of *VEGF* in gastric cancer has long been established [37] and in clinical trials, the second line use of this human monoclonal antibody (in combination with chemotherapy and as monotherapy) has shown a clear but modest survival advantage

Table 1
Intrinsic subtypes of CRC based on integrative molecular analysis.

Author	Subtype	Major subtype category	Subtype characteristics	Prevalence
TCGA	MSI/CIMP-H	MSI	Enriched for hypermutated tumours	30%
	CIN	Epithelial		30%
	Invasive	Mesenchymal		40%
Roepman et al.	A-type	MSI	Hypermutated, dMMR Good prognosis	22%
	B-type	Epithelial	MSS, BRAF WT, pMMR High proliferative activity Relatively poor baseline prognosis Most benefit from adjuvant chemotherapy	62%
	C-type	Mesenchymal	Undergone EMT Low proliferative activity Poor baseline prognosis No benefit from adjuvant chemotherapy	16%
De Sousa E Melo et al.	CCS1	Epithelial	Mainly left sided *KRAS* and TP53 + +	49%
	CCS2	MSI	Mainly right sided dMMR/MSI-H	24%
	CCS3	Mesenchymal	BRAF and *KRAS* + + Poor prognosis Upregulation of genes involved in matrix remodelling and EMT	27%
Marisa et al.	C1: CIN immune down	Epithelial	CIN + + + *KRAS* and TP53 + + Immune system and EMT down regulated	21%
	C2: dMMR	MSI	dMMR/CIMP + + + BRAF + +, *KRAS* + + Immune system and proliferation upregulated	19%
	C3: *KRAS* mutated	Epithelial	*KRAS* + + + Immune system and EMT down regulated	13%
	C4: Cancer stem cell	Mesenchymal	*KRAS* + + Proliferation down regulated EMT upregulated	10%
	C5: CIN Wnt up	Epithelial	CIN + + + *KRAS* and TP53 + + Wnt pathway upregulated	27%
	C6: CIN normal	Mesenchymal	CIN + + + Proliferation down regulated EMT upregulated	10%
Schlicker et al.	1.1	Mesenchymal	Activation of MAPK, TGFβ and calcium signalling	19%
	1.2	MSI	Activation of immune system-related pathways Highly enriched for MSI-H tumours	15%
	1.3	Mesenchymal	High expression of transporter genes	11%
	2.1	Epithelial	Activation of immune system-related pathways	23%
	2.2	Epithelial	High expression of genes on chromosomes 13q and 20q	32%
Budinska et al.	Surface crypt	Epithelial	*KRAS* + Upregulated top colon crypt, secretory cell and metallothioneins	26%
	Lower crypt	Epithelial	Upregulated top colon crypt, proliferation, Wnt Longest SAR	30%
	CIMP-H	MSI	MSI +, BRAF + Upregulated proliferation, immune, metallothioneins Shortest SAR	11%
	Mesenchymal	Mesenchymal	Upregulated EMT/stroma, CSC, immune	19%
	Mixed	Mesenchymal	P53 + Upregulated EMT/stroma, immune, top colon crypt, Chr20q, CSC	14%
Sadanandam et al.	Inflammatory	MSI	Comparatively high expression of chemokines and interferon-related genes Intermediate prognosis	18%
	Goblet	Epithelial	High mRNA expression of goblet-specific MUC2 and TFF3, Good prognosis May not benefit from adjuvant chemotherapy	14%
	Enterocyte	Epithelial	High expression of enterocyte-specific genes Intermediate prognosis	18%
	Cetuximab-sensitive transit amplifying	Epithelial	Higher levels of EGFR ligands known to predict cetuximab response Good prognosis	32%
	Cetuximab-resistant transit amplifying		Overexpressed FLNA (regulates expression and signalling of cMET receptor), cell lines more sensitive to cMET inhibition Good prognosis	
	Stem-like	Mesenchymal	High expression of Wnt signalling targets plus stem cell, myoepithelial and mesenchymal genes and low expression of differentiation markers Worst prognosis May benefit from adjuvant chemotherapy Most benefit from FOLFIRI	18%

[38,39]. It is evident however that even with the moderate impact of these two agents, the application of novel targeted therapies in GC has lagged behind other tumour types.

Integrative molecular subclassification of CRC and GC

In order to refine the molecular classification of CRC and to determine a standard and reproducible classification system, independent scientific groups have attempted to define the intrinsic subtypes of CRC using gene expression profiles (GEPs) [40–45]. Although there was hope that this will facilitate clinical translation and direct therapy, as has occurred in breast cancer for example [46], in reality there has been limited utility for these classifications above standard histopathological features. This is most likely due to the complex and overlapping findings identified between groups, whichever way they are defined. The findings of these studies are summarised in Table 1.

In 2012, The Cancer Genome Atlas (TCGA) Research Network produced a comprehensive integrative analysis of 224 colorectal cancer tumour samples and normal pairs in order to identify potential therapeutic targets [47]. Tumours were split into those that were hypermutated and those that were non-hypermutated. Initially, TCGA researchers considered colon and rectal tumours as separate entities due to their known anatomical and therapeutic differences. However, it was found that similar patterns of genomic alteration (copy number, expression profile, DNA methylation and miRNA changes) were seen in both types of tumours in the non-hypermutated group and thus they were subsequently analysed together at the genomic level.

TCGA analysis provided further confirmation of the pathways known to be deregulated in CRC. The vast majority of tumours in both groups had deregulated Wnt signalling, predominantly in *APC*, and inactivation of the TGF-β pathway was also seen, resulting in increased activity of *MYC*. New findings included recurrent mutations in *FAM123B, ARID1A*, and *SOX9* and very high levels of overexpression of the Wnt ligand receptor gene *FZD10* [47]. The *SOX9* gene is associated with intestinal stem cell differentiation and has not previously been shown to be implicated in CRC. It has been shown to facilitate β-catenin degradation [48] and its transcription is suppressed by Wnt signalling which is activated by extrinsic Wnt ligands. These findings suggest a number of potential therapeutic targets in CRC, namely Wnt signalling inhibitors and small molecule β-catenin inhibitors, which are beginning to show initial promise in pre-clinical models [49–51].

mRNA expression profiles separated the colorectal tumours into three clusters. One significantly overlapped with CIMP-H tumours and was enriched for hypermutated tumours, thereby representing a MSI/CIMP subgroup. The two other groups were representative of a CIN and an invasive phenotype subgroup [47].

Up to twenty seven molecular subtypes of CRC have been identified by these six studies but robust similarities do not exist between all of the studies, although correlation between two of the studies has been shown [52]. Two subtypes have been repeatedly identified (microsatellite instability enriched and high expression of mesenchymal genes) but full consistency amongst the others has not been achieved. More recently, in order to resolve these inconsistencies and integrate the data, the CRC research community formed an international consortium dedicated to large scale data sharing and analytics [5]. After analysing the independent transcriptomic-based classification systems (which comprised 18 CRC datasets and 4151 patients in total) and using unsupervised clustering techniques, four robust consensus molecular subtypes (CMSs) with distinguishing features were proposed. Tumours with mixed features (approximately 13%) were thought to represent a transition phenotype or intratumoral heterogeneity. Table 2 summarises the main biological, molecular, clinical and prognostic associations of the four consensus subtypes.

CMS1 encompasses mainly MSI tumours and this group is characterised by hypermutation, widespread hypermethylation, females, high rates of BRAF mutation, right-sided tumours and strong immune activation and infiltration with CD4+ T helper cells, CD8+ cytotoxic T cells and natural killer (NK) cells. The remaining three subtypes enable refinement of the 'non-MSI' CIN group of CRC tumours: CMS2 (canonical subtype), CMS3 (metabolic subtype) and CMS4 (mesenchymal subtype). CMS2 and CMS4 subgroups are similar in that they both represent MSS and low levels of gene hypermethylation. They differ however at the genomic level with CMS2 showing marked upregulation of Wnt and *MYC* downstream targets and higher expression of oncogenes *EGFR*, *ERBB2*, Insulin-like growth factor 2 (*IGF-2*), insulin receptor substrate 2 (*IRS-2*) and transcription factor hepatocyte nuclear factor 4α (*HNF4α*). CMS4 tumours showed upregulation of genes associated with epithelial-mesenchymal transition such as transforming growth factor β (*TGF β*) and integrins. This subgroup also shows prominent stromal invasion and angiogenesis and correlates with poorer outcomes. CMS3 tumours were found to be enriched for multiple metabolism signatures in keeping with the overexpression of activating *KRAS* mutations in this group which have been linked to prominent metabolic adaptation in CRC.

Within the four CMS subtypes, no clear single molecular event or genetic aberration was found to be limited to one subtype and no clear associations were drawn from integrative genomic analysis either. This highlights the concept that in CRC, tumours with assumed driver events still have varied biology which is reflected in the poor genotype-phenotype correlation in this disease as well as the heterogenous drug responses [5].

A significant breakthrough in the molecular characterisation of GC was achieved through the work of TCGA network and its analysis of 295 untreated GC samples from a global population [53], from which a new classification system describing four molecular subtypes was outlined (Table 3). However, the majority of cases (approx. 75%) in TGCA analysis were still derived from a western population whose clinical course and biological characteristics differ from those in the Far East where the majority of global GC cases arise. Recent data from the Asian Cancer Research Group (ACRG) [54] has allowed the global applicability of TCGA classification to be further explored. The ACRG used gene expression data to characterise 300 Korean surgical specimens from gastric resections. Four subtypes were again defined:

1. Microsatellite stability (MSS)/epithelial-to-mesenchymal transition (EMT)
2. Microsatellite instability (MSI)
3. MSS/p53+
4. MSS/p53−

These subtypes share some similarities with the TCGA classification system, for example in their ability to identify tumours with MSI. There is also considerable overlap between the TCGA GS, EBV positive, and CIN subtypes and the ACRG MSS/EMT, MSS/p53+ and MSS/p53− subtypes respectively. This gives validity to both data sets. Differences do exist however in the prevalence of certain mutations in particular subgroups: *CDH1/RHOA* mutations for example are known to be common in GS tumours in the TCGA classification but are not prevalent in the ACRG MSS/EMT subgroup. There are also differences in the clinical phenotypes within each data set (higher proportion of diffuse cancer and stages III/IV disease in ACRG, higher proportion of gastro-oesophageal junction (GOJ) tumours in TCGA) which may explain some differences in underlying driver mutation prevalence. A significant advantage of the ACRG analysis is the availability of long-term follow up data which has allowed prognostic associations to be made whereas this is lacking from TCGA analysis.

These two classifications systems have provided a much needed step forward for GC, offering the potential to identify many therapeutic targets. However, more work is needed to clarify which of the many mutations identified are true driver mutations and worthy of therapeutic exploration, as well as to describe key epigenetic and transcriptome alterations. Whilst the ACRG classification goes some way to

Table 2
The four consensus molecular subtypes of CRC.

	CMS1 MSI immune 14%	CMS2 Canonical 37%	CMS3 Metabolic 13%	CMS4 Mesenchymal 23%
Genomic characteristics	MSI high CIMP high Hypermutation SCNA low	SCNA high	Distinctive profile: Mixed MSI status SCNA low CIMP low	SCNA high
	Overexpression of proteins involved in DNA damage repair Widespread hypermethylation status	Higher chromosomal instability	Intermediate levels of gene hypermethylation	Higher chromosomal instability
Molecular features	*BRAF* mutations Activation of RTK and MAPK pathways		*KRAS* mutations Activation of RTK and MAPK pathways	
	Immune infiltration and activation Strong activation of immune evasion pathways	Wnt and MYC activation	Metabolic deregulation	Stromal infiltration TGF-β activation Angiogenesis
Clinical features	Females Right sided tumours Higher grade	Left sided tumours		More advanced stages
Prognostic features	Better relapse- free survival Worse survival after relapse	Better survival after relapse		Worse relapse-free and overall survival

linking identified genetic changes with prognosis, more effort needs to be deployed overall into determining the functional and clinical significance of the molecular findings.

Clinical and prognostic associations of the CRC consensus molecular subtypes

Prognostic associations of the CMSs have been explored by using the subset of patients within the datasets which was derived from the large adjuvant chemotherapy trial PETACC-3 [55] as well as by using other combined datasets. CMS1 tumours exhibit a good prognosis but poor survival following relapse, in keeping with data already described for MSI tumours with mutated BRAF. Conversely, CMS4 tumours show worse overall and relapse-free survival which persists after univariate and multivariate adjustment for MSI status, *BRAF* and *KRAS* mutations. CMS2 and CMS3 subgroups display intermediate survival with superior survival after relapse seen in the CMS2 subgroup [56].

The prognostic associations of the four CMSs in the advanced disease setting, as well as their associations with biological therapies, have also been explored via retrospective analysis of large clinical trial datasets. Three studies in advanced disease have shown that CMS2 tumours exhibit the best prognosis and CMS1 tumours the worst [57–59]. These studies show that the prognostic effect of the CMS subtypes in advanced disease are highly consistent and also stage-dependent given the concordance with survival-after-relapse from the early stage cohorts. In the CALGB 80405 dataset, patients with tumours within CMS1 subtype who received bevacizumab had significantly longer OS compared to those who received cetuximab. Conversely, patients with CMS2 tumours who received bevacizumab had a trend towards shorter OS than those who received cetuximab [59]. In the first-line advanced disease MAX study, CMS2, and possibly CMS3, tumours preferentially benefited from the addition of bevacizumab to capecitabine-based chemotherapy [57]. The differing results from these retrospective analyses may be due to the lack of comparability between the studies as the MAX study had no mutational selection criteria and the treatment backbones within the studies also differed. All of these results are hypothesis-generating and require further validation but this data suggests that the relative efficacy of biological therapies may differ according to CMS subtype. Given that bevacizumab is approved and widely used worldwide in CRC but not all patients derive clinical benefit from it, there is an urgent need to identify a predictive biomarker for anti-angiogenic agents in CRC in order to guide management decisions.

Tumour sidedness has recently been recognised as increasingly

Table 3
The four molecular subtypes of gastric cancer defined by TCGA analysis.

Subtype	EBV	Microsatellite instability (MSI)	Chromosomal instability (CIN)	Genomic stability (GS)
Main subtype characteristics	High EBV burden DNA promoter hypermethylation	High mutation rates Promoter hypermethylation	High somatic copy number aberrations	low copy number aberrations
Approximate frequency	10%	20%	50%	20%
Anatomical distribution	Fundus/Body	Body	Cardia/GOJ	Antrum
Clinicopathological features	Males	Females Older age	Intestinal type	Younger age Diffuse type Worse prognosis
Examples of Gene Alterations	EBV-CIMP PIK3CA mutation (80%) PD-L1/2 overexpression JAK2 amplification ERBB2 amplification CDKN2A silencing Immune cell signalling	Gene hypermutation Gastric-CIMP MLH1 silencing Mitotic pathways	TP53 mutations RTK-RAS activation e.g. CDK6, MET	RHOA mutation CDH1 mutation CLDN18-ARHGAP fusion
Potential therapeutics	PI3K inhibitors AKT inhibitors Dual PI3K-mTOR inhibitors JAK2 inhibitors Immunotherapy	Immunotherapy	Anti-HER2 therapy (Trastuzumab, dual blockade, TDM-1) CDK4/6 inhibitors (Palbociclib) MET blockade FGFR inhibitors	AURKA inhibitors PLK1 inhibitors

important to help distinguish the underlying molecular differences between CRC tumours which can determine prognosis. CMS1 tumours are predominantly right sided and CMS2 tumours are predominantly left sided. Improvements in OS with cetuximab in left sided tumours has been confirmed retrospectively in the FIRE-3 (FOLFIRI + cetuximab/bevacizumab first line) and CRYSTAL (FOLFIRI + cetuximab first line) datasets [60]. No significant differences in survival between left and right sided tumours were identified with bevacizumab. Further to this, a meta-analysis of six randomised trials, including the two mentioned above, also demonstrated an improvement in PFS and OS for left sided tumours treated with anti-EGFR antibodies compared to no significant benefit for right sided tumours [61]. Taken together, bevacizumab can be useful for all CRCs regardless of sidedness but anti-EGFR therapies appear useful only in left sided tumours in the first-line setting and this recommendation has now been incorporated into the NCCN clinical practice guidelines in oncology. Key biological features of tumours of differing side may partially underpin this pattern of response: right sided tumours show reduced expression of the *EGFR* ligands amphiregulin (*AREG*) and epiregulin (*EREG*) which is linked to hypermethylation of the ligand's promoter regions [62]. Conversely, left sided tumours often overexpress *EGFR* ligands and harbour amplifications of *EGFR* and *IRS2* which are markers of cetuximab sensitivity [63].

As described above, the prognostic value of the CMSs in CRC appears to be consistent across studies. However, it appears that certain predictive criteria may not be distinguishable under this classification: a separate multivariate analysis of the CALGB 80405 dataset showed that sidedness of the primary tumour (right versus left) was still an independent prognostic factor over and above CMS subtype [64]. In order for CMS subtyping to be a clinically useful tool for guiding patient management, prospective studies are required to robustly demonstrate the association of this molecular subclassification with treatment and survival outcomes.

Microsatellite instability in CRC and GC

Sporadic CRC MSI-H tumours are more likely to be right-sided (proximal), poorly differentiated, mucinous, associated with tumour infiltrating lymphocytes (TILs) and have higher rates of BRAF mutation (approximately 50%) [11,65]. Microsatellite stable (MSS) tumours are more frequently left sided (distal) and have higher rates of *KRAS* mutation. MSI status has been found to be an important prognostic and now predictive marker in CRC. It has been shown that MSI-H tumours have improved stage-adjusted survival (Stage I-III) compared to MSS tumours treated with surgery alone [66]. In metastatic disease, studies have however shown a worse prognosis of MSI-H tumours but this is driven by their association with BRAF mutations [67].

MSI status also inversely predicts for benefit of adjuvant 5-FU chemotherapy as MSI-H tumours do not derive the survival benefit that MSS tumours do with adjuvant 5-FU chemotherapy [68,69]. This is postulated to be due to the chemotherapy dampening the immune response to the tumour that characterises this phenotype. Current clinical practice should incorporate the use of MSI status testing to inform decisions regarding adjuvant chemotherapy in stage II colon cancers.

The utility of MSI testing in advanced CRC to direct treatment options has recently become of major importance, relating to the significant benefit of the use of PD-1 blocking immunotherapy agents. Le et al. [70] showed an immune-related objective response rate (ORR) of 40% and immune-related PFS rate of 78% for MMR deficient colorectal tumours treated with pembrolizumab compared to 0% and 11% respectively for MMR proficient tumours.

As identified by TCGA analysis, a significant proportion of gastric cancers exhibit MSI. The association between MSI-H CRC tumours and improved prognosis and lack of benefit to adjuvant chemotherapy has been reproduced in GC. Smyth et al. [71] analysed MMR and MSI status in 303 samples from the Medical Research Council Adjuvant Gastric Infusional Chemotherapy (MAGIC) study in operable gastric and oesophagogastric junctional adenocarcinomas [72] and found that MSI-H tumours exhibited better survival than MSS tumours and did not derive benefit from peri-operative chemotherapy. This predictive utility of MSI testing in early GC was also confirmed in the Eastern population by post-hoc analysis of the adjuvant CLASSIC study [73]. As peri-operative or adjuvant chemotherapy is the standard of care in early GC, these results could provide a method of selecting patients for treatment, just as for adjuvant chemotherapy in CRC patients, thereby sparing those patients who will not derive benefit from the toxicities of chemotherapy.

Emerging immune subtypes of CRC and GC

In the current evolving era of cancer immunotherapy, the spotlight has now shifted onto the tumour microenvironment (TME) and the effect of tumour immune infiltrate on clinical outcome. In CRC, comprehensive analyses of immune phenotypes have been carried out recently with immune-based classifications, such as Immunoscore [74], displaying evidence of promising clinical utility.

Becht and colleagues [75] have shown that the composition of the TME varies between each CMS subgroup. CMS1 and CMS4 subtypes both display high levels of infiltrating immune cells but these subtypes are opposite in their functional orientation [76]. It is now well established that MSI-H CRC (enriched in CMS1 subtype) is associated with a high mutational burden, high levels of TILs and subsequent increased responsiveness to immune checkpoint blockade [77]. Another cluster of tumours displaying an 'inflamed' immune phenotype has been identified and is characterised by upregulation of immunosuppressive factors such as *TGFβ* and *CXCL12*, and high expression of chemokine encoding genes [78]. Many of these features overlap with CMS4 tumours and suggest the creation of an immune evasive microenvironment driven by tumour-derived stromal cells, which could help explain the poor outcomes of this subtype. Conversely, the majority of tumours in the CMS2 and CMS3 subtypes, which display MSS, are non-hypermutated and possess a microenvironment lacking in TILs and immunoregulatory cytokines, suggesting they are likely to be poorly immunoresponsive ('non-inflamed') [75]. These three immune phenotypes are characterised by differences in the tumour cells themselves as well as stromal cells in the TME (the immune contexture) [79]. As CMS2 and CMS3 tumours comprise half of all CRC, it is imperative to fully understand the mechanisms of immune evasion in these cancers to aid our understanding of how to prime the microenvironment of these tumours to render them more responsive to immunotherapeutic agents (non-inflamed to inflamed). As such, early clinical and translational trials in CRC are increasingly exploring combination regimens of immune 'priming' agents alongside immunotherapy drugs in an attempt to achieve this.

As seen in CRC, the MSI subgroup of GC has also been shown to be highly immunoresponsive [70]. In addition, TCGA-defined EBV subgroup, which is mutually exclusive to MSI, is considered to have a rich immune infiltrate and therefore also regarded as highly immunoresponsive. Certainly, dramatic responses have been observed in these two subtypes to PD-1 blockade with pembrolizumab in the Korean population (ORR 85.7% in MSI-H and ORR 100% in EBV positive advanced GC) [80]. Activity of PD-1 inhibitors in advanced GC has been promising and has led to Food and Drug Administration (FDA) approval of pembrolizumab in pre-treated patients with a combined positivity score of PD-L1 expression of $\geq 1\%$ [81] based on results of the KEYNOTE-059 study [82]. PD-L1 expression on cancer cells has clinical utility as a predictive biomarker to immunotherapy in other cancers, predominantly non-small cell lung cancer (NSCLC) [83], although challenges still arise, in terms of differential expression of PD-L1 within tumours and no standardised definition for overexpression, in regards to its use as a robust biomarker [84]. In addition, PD-L1 positivity in GC is predominantly due to expression on infiltrating immune cells in the stroma rather than on tumour cells which is different to that observed

in NSCLC [85]. Although MSI-H and EBV positive gastric cancers display higher levels of PD-L1 expression [86], responses to immunotherapy have been seen in patients who are PD-L1 negative too [87,88]. The data above suggest PD-L1 expression alone is not a robust tool at predicting response to immunotherapy in GC.

Over the last decade, our understanding of immune modulation has greatly improved and immunotherapy is rapidly evolving in GI malignancies with more promise being seen in GC over CRC thus far [89]. Clinical trials are ongoing in both tumour types to explore various immunotherapeutic approaches at different treatment stages but the search for robust predictive biomarkers alongside these studies continues to be of vital importance to gain the maximum benefit of this therapeutic approach. As explained above, subsets of patients with CRC and GC who are most likely to respond to immunotherapy have been identified but they do not represent the majority of patients with these diseases.

Integration of molecular profiling into routine clinical practice

In generating the large amounts of data that molecular analysis yields, the challenge remains in both upper and lower GI cancers to find a simple, clinically relevant subclassification that is both prognostic and predictive, helping patients and clinicians to make treatment choices and explore rationale therapeutic targets and combination therapies. Integrating molecular characteristics into clinical trial design is essential to enable true clinical translation and precision medicine.

Despite initial enthusiasm that the CMS subtypes of CRC would easily identify patients for appropriate clinical trials, this classification system is based on complex transcriptomic analysis with overlaid genomic information. The reproducibility of this classification in the clinical setting is therefore challenging although work continues on simplifying testing, ideally to a high throughput, relatively cheap technology such as IHC analysis. Trinh and colleagues have proposed an IHC based classifier derived from tissue microarrays from 1076 CRC samples [90]. Five markers (*CDX2, FRMD6, HTR2B, ZEB1* and *KER*) along with MSI status were used to demonstrate 87% concordance with transcriptome-based classification and this was validated in three separate datasets, although prospective validation is required.

Challenges also lie in the provision of sufficient viable tumour within biopsies or resection specimens (particularly after neoadjuvant therapies as these increasingly become standard of care for both upper and lower GI cancers). Extraction of good quality specimens for transcriptome analysis from FFPE tissue is a barrier for real time use in clinical practice, where obtaining fresh tissue remains a challenge outside of dedicated research resourcing. Tumour heterogeneity and temporal changes during the course of disease are also both ongoing challenges, as with all tumour types, although there is great optimism that liquid biopsies will overcome the 'tissue issue'.

Integration of molecular profiling into clinical trial design

The potential clinical utility of CMS classification has been extensively evaluated in resection specimens but has not been widely assessed in FFPE biopsy specimens, although there is some initial evidence of disappointing results, primarily due to stromal intratumour heterogeneity [91]. This may limit the applicability of the CMS subtypes into prospective molecularly-guided clinical trial design which utilises profiling of pre-treatment tumour biopsies for patient stratification. More effort is thus required to overcome this as biomarker and drug co-development is essential for the success of biomarker-guided therapies.

It is clear that the traditional single-question molecularly unselected studies are no longer optimal given our current knowledge of tumour heterogeneity. The move towards molecularly selected, adaptive and innovative clinical trial design is paramount and will rely on international collaboration, between investigators as well as pharmaceutical companies, as the subsets become more refined. An example of a new paradigm of clinical trial design incorporating the molecular heterogeneity of CRC is the currently recruiting FOCUS4 study: a molecularly stratified randomised clinical trial incorporating a multi-arm, multi-stage (MAMS) design. The adaptive design of the trial allows for treatments to be discontinued if they do not show enough of an effect and for new biomarkers and novel agents to be incorporated into the study if needed. However, the running of trials such as this is relatively difficult for a variety of reasons including small numbers in each cohort, lack of specific targets, difficulty coordinating new therapeutics from multiple industry partners, requirement to open in multiple countries with different models for funding, ethics approval and so on.

Following the failure of multiple targeted therapy trials in advanced GC [92–94], there is an urgent need for more adaptive and innovative clinical trial design in this disease. A phase III molecularly-selected study of the anti-CLDN18.2 antibody IMAB362 alongside first-line chemotherapy in advanced claudin18.2 expressing gastric/GOJ adenocarcinoma, as defined by TCGA subtype GS, is underway based on promising results of the phase II study [95]. However this trial is estimated to take almost three years to accrue and four and a half years to complete, delaying drug approval and routine access for patients.

Conclusion

Much work has been undertaken to further improve our understanding of the underlying molecular mechanisms implicated in the development and progression of colorectal and gastric cancers. It is clearly evident that there is marked heterogeneity within these cancers and simple histopathological classification is far too inept to predict therapeutic response. What is emerging is a complex landscape of genomic instability and intricate molecular changes that require deeper understanding and stratification in order to fulfil the vision of precision medicine or true personalised therapy. Fundamental to this is clinical trial design incorporating biomarker stratification for molecularly rational therapies, as highlighted by the successful integration of this strategy into current clinical trial design. Highly translational studies incorporating biospecimens and sequential biopsies are needed in order to identify markers of response, prognosis and also treatment resistance.

The vast amounts of data being generated from molecular classification systems are hypothesis-generating but the application of this data to clinical practice remains a challenge. Widespread reproducibility and confirmation of clinical utility of molecular stratification systems are currently key questions which are beginning to be addressed. Molecular classification systems in CRC and GC need to continue to be applied to large clinical trial registry datasets as well as prospectively stratified in large clinical trials in an attempt to fully validate the molecular subtypes and, more importantly, to identify robust predictive biomarkers which can be utilised in order to rationalise drug development.

Conflict of interest

Avani Athauda: no conflict of interest.

Eva Segelov: has received conference sponsorship/travel support from Ipsen and Amgen and sits on the advisory board for Ipsen.

Zohra Ali: no conflict of interest.

Ian Chau: sits on the advisory board for Eli Lilly, Bristol Meyers Squibb, MSD, Bayer, Roche, Merck-Serono and Five Prime Therapeutics. He has received research funding from Eli Lilly, Janssen-Cilag, Sanofi Oncology and Merck-Serono and has received an honorarium from Eli Lilly

Acknowledgements

AA, ZA and IC would like to acknowledge National Health Service

funding to the National Institute for Health Research Biomedical Research Centre at the Royal Marsden NHS Foundation Trust and The Institute of Cancer Research.

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