Molecular classifications of gastric cancers: Novel insights and possible future applications

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Abstract

Despite some notable advances in the systemic management of gastric cancer (GC), the prognosis of patients with advanced disease remains overall poor and their chance of cure is anecdotic. In a molecularly selected population, a median overall survival of 13.8 mo has been reached with the use of human epidermal growth factor 2 (HER2) inhibitors in combination with chemotherapy, which has soon after become the standard of care for patients with HER2-overexpressing GC. Moreover, oncologists have recognized the clinical utility of conceiving cancers as a collection of different molecularly-driven entities rather than a single disease. Several molecular drivers have been identified as having crucial roles in other tumors and new molecular classifications have been recently proposed for gastric cancer as well. Not only these classifications allow the identification of different tumor subtypes with unique features, but also they serve as springboard for the development of different therapeutic strategies. Hopefully, the application of standard systemic chemotherapy, specific
targeted agents, immunotherapy or even surgery in specific cancer subgroups will help maximizing treatment outcomes and will avoid treating patients with minimal chance to respond, therefore diluting the average benefit.

In this review, we aim at elucidating the aspects of GC molecular subtypes, and the possible future applications of such molecular analyses.

**Key words:** Molecular biology; Immunotherapy; Gastric cancer; Classification; Targeted therapy

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**Core tip:** TCGA individuates four molecular subtypes: Chromosomal instability, microsatellite instability, genomically stable and Epstein-Barr virus positive tumors. Asian Cancer Research Group classification partially overlaps with the previous one. Although not prospectively validated, these novel classifications suggest that different subtypes of gastric cancer might be treated with specific therapeutic strategies in the near future.

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**INTRODUCTION**

Gastric cancer (GC) is among the most common malignancies worldwide and the second leading cause of cancer related deaths[1]. In fact, it represents the fifth most commonly diagnosed cancer (6.8% of oncologic diagnoses) resulting in an annual estimated incidence of 18 cases out of 100000 individuals among men and 9 out of 100000 for women[2].

The mainstay of first-line therapy for GC is still represented by a chemotherapy backbone composed by platinum compounds and fluoropyrimidines resulting in a median overall survival (OS) of about 11 mo. Still, the disappointing 5-year survival rate is estimated to be about 25%-30% and slightly higher for some Asian experience. Historically, many attempts have been made in order to re-classify gastric cancer with the aim of clustering some new subgroups that could have different prognostic and predictive value: Anatomical classification (Borrmann classification and Siewert and Stein classification), histological classification (WHO classification and Lauren's classification), and extent of disease (early gastric cancer vs advanced cancer).

The first effective molecular novelty came from the TOGA trial which demonstrated a significant improvement in OS with the addition of trastuzumab to chemotherapy when compared to chemotherapy alone in patients with HER2 overexpressing GCs (13.8 mo vs 11 mo, respectively; \( P = 0.046 \))[3]. Another clue to the “heterogeneity theory” comes from the observation that Asian patients demonstrate different pattern of disease and outcomes if compared to the Caucasian western population included in the largest trials.

Nowadays, with mounting biological information available, almost every solid cancer type is considered as a "collection" of multiple very molecularly heterogeneous diseases. Very important advances have been made in the molecular classification of breast cancers[4], lung tumors (by the identification of some tyrosine-kinase-inhibitor targetable subtypes), colorectal adenocarcinomas (predictive and prognostic classes sorted by mutations in RAS and BRAF genes), and malignant melanoma (identification of BRAF codon 600 mutation).

Nevertheless, the poor anatomical and molecular selections of GC patients entering clinical trials have potentially limited the effect of many therapeutic agents including chemotherapy, antiangiogenic drugs and the newly tested immune-modulators. In fact, the benefit of those drugs may have been diluted when tested in the overall population. Recently something has changed the way of thinking GC starting from the TCGA group publication appeared in 2014[5].

A more profound understanding of the molecular clustering of stomach cancer could give us the chance to obtain new insights into prognostic and predictive categorization of this cancer and could definitely provide the scientific knowledge for developing modernly conceived clinical trials that could maximize the effect of novel agents in the proper patient population, avoiding the use of costly drugs in non-stratified populations.

Finally, the aim of this review is to give a general picture of the current knowledge of the emerging molecular classification of GC and to explore the new possibilities connected to the latest discoveries made on the extreme heterogeneity of this disease.

**THE IMPORTANCE AND LIMITATIONS OF MOLECULAR CLASSIFICATIONS**

The first attempt to generate a comprehensive molecular classification for GC was made in 2013 by Singapore Researchers[6]. They identified three main types of gastric cancer, namely proliferative (characterized by high genomic instability and TP53 mutation), metabolic (more sensitive to S-FU therapy) and mesenchymal (stem cell-like tumors sensitive to PIK3CA-mTOR pathway inhibitors), based on genome expression. Soon after the TCGA research group published a classification dividing GCs into four main subgroups clustered on the basis of six different molecular biology approaches: Copy number variation (CNV) analysis, exome sequencing analysis, DNA methylation profile, mRNA sequencing, micro-RNA (miRNA) sequencing and reverse phase protein array[2]. The result...
is the subdivision of GC into four genomic subtypes: Epstein-Barr virus (EBV) positive cancers (9% of all gastric tumors with frequent PIK3CA mutation and PD-L1/PD-L2 overexpression), Microsatellite Instability tumors (MSI, representing 22% and hypermutated), chromosomal instability (CIN, 50%, predominantly junctional, TP53 mutated with RTK-RAS activation, with a high rate of CNV) and Genomically Stable (GS, 20%, presenting mutation in motility and adhesion molecules). Specific TCGA molecular subtypes are represented in Figure 1.

In the meantime, the Asian Cancer Research Group (ACRG) too proposed a novel molecular classification[7], and the resulting taxonomy divided GCs into: Mesenchymal subgroup (MSS/EMT, characterized by hallmarks of epithelial-to-mesenchymal transition), Microsatellite Instability subgroup (MSI), Microsatellite Stable TP53 positive (MSS/TP53+, somehow overlapping with EBV type of TCGA classification) and Microsatellite Stable TP53- tumors (MSS/TP53-, overlapping with CIN by TCGA).

These novel classifications create a new paradigm in the definition of cancer biology and allow the identification of relevant genomic subsets by using different techniques such as genomic screenings, functional studies and molecular or epigenetic characterization. However, some limitations should also be openly recognized. First, these classifications are based on a highly complex methodology and currently they should not be replicated in standard laboratories lacking in the utmost technologies. Attempts towards simplification are ongoing although results may not fully capture the underpinning complexity of the disease. Second, these classifications lack of a prospective validation on a large scale, including patients with different ethnicity and age. Third, the two proposed classifications have more differences than similarities; in particular, they are different in terms of demographics, baseline molecular mechanisms, driver genes, and association with prognosis. Moreover, there are notable dissimilarities in the distribution of Lauren's diffuse subtype among the different subgroups. Since different molecular subgroups may be identified across a number of independent gene expression profile studies, a collaborative international effort is warranted to aggregate a consensus classification. Fourth, the follow-up of included patients is limited, factor that may decrease their prognostic power, and subgroups were evaluated on resected specimens, with different prevalence of subgroups between localized, locally advanced and advanced settings. Fifth, both classifications insist on epithelial cells, but none of them take into account the active, nonmalignant stromal cells. Actually, not only gene expression profiles deriving from stromal tissues may influence assignment to a specific molecular category, thus creating interpretative troubles[8], but also novel stromal-based distinctive signatures have been proposed and related to the predominant cancer phenotype[9].

**GC WITH CHROMOSOMAL INSTABILITY**

CIN subtype represents approximately 50% of GCs[10] and it mostly occurs in the esophagogastric junction (EGJ)/cardia. CIN GC is related to intestinal type histology,
to copy number gains of chromosomes 8q, 17q and 20q, while, gains at 12q and 13q are associated with diffuse GC\cite{[27]}. Interestingly, CIN showed elevated frequency in the EGJ/cardia, as demonstrated in TCGA characterization (65\%, \textit{P} = 0.012). CIN is characterized by somatic mutations at cytogenetic level, particularly involving loci that control mitotic checkpoints, thus gatekeeper and caretaker genes implicated in carcinogenesis. CIN comprises both altered DNA copy number and structural abnormalities in some chromosomal regions. Those alterations could result in gain or loss of whole chromosomes\cite{[11]} (aneuploidy), non-reciprocal translocations, amplifications, deletion or the loss of one allele with loss of heterozygosis. Altogether, CIN results in the loss or gain of function of some “key genes”, including oncogenes and tumor suppressor genes that may be efficaciously targeted by specific inhibitor molecules\cite{[13]}. Notably, CIN GC is enriched in mutations in 7p53 gene and receptor tyrosine kinases (RTKs), furthermore it shows amplifications of cell cycle genes (Cyclin E1, Cyclin D1, and Cyclin-dependent kinase 6)\cite{[19]}.

Evaluation of the biological characteristics among CIN cancers demonstrated that 7p53 mutations occurs in 71\% of GCs\cite{[5]}. Furthermore, CIN also display amplification in oncogene pathways such as RTK/RAS/MAPK signaling, including HER2, BRAF, epidermal growth factor (EGFR), MET, FGFR2, RAS\cite{[5,15]}

A recent work reviewed the pathogenic and molecular similarities between gastric intestinal-type adenocarcinoma and esophageal adenocarcinoma (EAC)\cite{[16]}, suggesting that treatment of EAC should recall that of gastric adenocarcinoma rather than being similar to the approach used for upper esophageal cancers (mostly squamous). In fact, not only EAC may arise from progenitor cells deriving from the cardia of the stomach but also the majority of EAC express a chromosomal instability that closely resembles the one found in CIN GC. All these findings suggest both the need for better subtyping esophageal cancers and the opportunity of developing specific therapeutics strategies in this disease as well.

\textbf{HER2}

The proto-oncogene HER2 is a member of the EGF receptor family with tyrosine kinase activity. It is known that HER2 positivity may vary depending on the primary tumour location as well as on the histotype of gastric cancer. Indeed, \textit{HER2} overexpression/amplification is detected in more than 30\% of the tumours arising from the gastroesophageal junction whereas less than 20\% of tumours in the gastric body are \textit{HER2}-positive. In addition, intestinal and diffuse histotype display a rate of \textit{HER2} positivity of 34\% and 6\% respectively\cite{[17]}. \textit{HER2} plays a key role in a large number of cellular processes, including cell differentiation, proliferation, motility and signal transduction. After the combination of chemotherapy and \textit{HER2} targeted therapy with trastuzumab had defined a new standard of care for \textit{HER2}-positive metastatic GC\cite{[18,19]}, other \textit{HER2} inhibitors were tested.

Lapatinib, a multi-kinase inhibitor, was evaluated in two randomized phase III trials enrolling GC patients with advanced disease. The LOGiC trial tested the efficacy of lapatinib in combination with capecitabine plus oxaliplatin given upfront. The addition of lapatinib did not significantly increase OS (12.2 mo vs 10.5 mo, hazard ratio (HR) 0.91, P = 0.349), although progression-free survival (PFS) was longer (6.0 mo vs 5.4 mo, HR 0.82, P = 0.0381) and objective response rate (ORR) was higher (53\% vs 39\%, P = 0.0031) in the lapatinib arm\cite{[19]}. The TyTAN trial randomized 261 Asian patients to receive lapatinib plus paclitaxel or paclitaxel alone in second-line treatment. Disappointingly, no marked survival differences between treatment groups were noted: Median OS (11.0 mo vs 8.9 mo, P = 0.1044) and PFS (5.4 mo vs 4.4 mo, P = 0.2441). Overall, 15 patients (6\%) had previously received trastuzumab, 8 in the lapatinib/paclitaxel arm and 7 in the paclitaxel alone arm\cite{[20]}.

\textit{JACOB}, a large randomized phase III trial designed to test the efficacy of pertuzumab in combination with trastuzumab and standard chemotherapy (cisplatin plus fluoropyrimidine) has recently completed the accrual\cite{[21]}. Results of the trial are eagerly awaited. Novel anti-\textit{HER2} drugs have been developed to try to overcome secondary trastuzumab resistance, as in the case of trastuzumab-emptansine (T-DM1). Data from phase III GATSBY trial were recently presented concluding that TDM-1 did not improve patients’ outcome compared to second-line taxanes at the 2015 clinical cut-off\cite{[22]}.

The majority of gastric cancer patients who achieve an initial response to trastuzumab-based regimens develop resistance within 7 mo\cite{[23]}. These unsatisfactory results may be attributed to primary (de novo) or secondary (acquired) resistance to the \textit{HER2}-targeted therapy. Therefore, as it happened for breast cancer, the onset of trastuzumab resistance has been investigated also in gastric cancer, showing several molecular mechanisms underlying the acquired resistance to \textit{HER2} inhibitors\cite{[24]}. Lee \textit{et al}\cite{[25]} identified that \textit{HER2}-amplified GC patients have diverse pattern of various concurrent molecular events. Zuo \textit{et al}\cite{[26]} employed the human gastric carcinoma cell line NCI-N87 with high \textit{HER2} expression to create trastuzumab-resistant NCI-N87/TR cells by stepwise exposure to increasing doses of trastuzumab. They showed that activation of the PI3K-AKT signalling pathway downstream of \textit{HER2} was one of the major mechanisms leading to resistance of NCI-N87/TR gastric cancer cells to trastuzumab, which was probably associated with \textit{PTEN} gene down-regulation and mutation, as well as with over-activity of the IGF-1R signalling pathway\cite{[26]}. The study conducted by Piro \textit{et al}\cite{[27]} identified the FGFR3/AKT axis as an escape pathway responsible for trastuzumab resistance in gastric cancer, indicating that the inhibition of FGFR3 could be a potential strategy to modulate this resistance. Recently, Arienti \textit{et al}\cite{[28]} explored the role of the IQ-domain GTPase-activating protein 1 (IQGAP1), a multifunctional scaffold protein, which interacts with diverse proteins to regulate cell adhesion and cell migration. IQGAP1 governs \textit{HER2}-expression, phosphorylation and signalling in breast cancer cell lines\cite{[29]}, it is overexpressed in aggressive form.
The failure of anti-EGFR monoclonal antibodies in advanced gastric intestinal-type adenocarcinoma. In this work, with the aim of elucidating the prevalence of anti-EGFR overexpression/amplification, the HER2 overexpression/amplification and the combination of the previous two. Interestingly, EGFR overexpression was more frequent in intestinal-type GCs (32.7% of the specimens) and its genomic amplification was demonstrated in 14.1% of the patients. It has also been shown that EGFR amplification was associated to a deeper tumor invasion (pT3-4 vs pT1-2, OR 2.15, P = 0.029). This unfavourable clinical feature correlated also to a shortened time to cancer recurrence (P = 0.026) and cancer specific survival (P = 0.033). Furthermore, HER2 overexpression/amplification has been shown to be less frequent when compared to EGFR overexpression/amplification and EGFR/HER2 co-amplification (3.6% of the cases), indicating that these different populations may bear specific genomic alterations potentially approachable with different treatments. All these data strongly suggest that modern trials should be designed with a careful stratification according to EGFR amplification to properly assess the clinical effectiveness of anti-EGFR drugs in GC patients.

RAS and BRAF
KRAS mutation occurs in less than 5% of GC and may have a negative prognostic value in GC patients. KRAS activates critical pathways involved in carcinogenesis and tumor progression, such as PI3K-Akt, RAF, MEK-extra-cellular signal regulated kinase and NF-kB. However, no target therapies are currently approved for this molecular aberration[41]. Other drugs, such as MEK inhibitors were tested in KRAS mutated cancer cell lines with promising results. Since preclinical study suggested that the combination of MEK-inhibitors and PI3K or BCL-XL inhibitors may be efficacious in KRAS mutant lung cancer patients[42], it would be intriguing to evaluate MEK inhibitors in monotherapy or in combination with PI3K inhibitors or BCL-XL in GC patients who carry this mutation. In GC patients, BRAF mutations are rare (2.2% in TCGA database) and are mostly represented by BRAF V599M[43]. The role of this mutation in GC is yet to be assessed.

FGFR2
FGFR2 amplification is associated with tumor cell proliferation and survival of GC cell lines and indicates poor prognosis. In the TCGA classification, approximately 9% of CIN GC patients had FGFR2 gene amplification. Several drugs and studies targeting this mutation are ongoing[44]. A phase II randomized trial is evaluating the activity of AZD4547 (a FGFR 1-2 and 3 inhibitor) compared to paclitaxel in second-line treatment. Other ongoing trials are testing dovitinib in FGFR2 amplified GC patients or in combination with docetaxel[45].

C-MET
Mesenchymal epithelial transition factor (MET) alteration was rarely observed in GC (8%)[41]. MET is an RTK that interacts with its native ligand HGF. Deregulated expression of C-MET in GC has been related to worse
prognosis. In fact, the HGF/c-MET signal is involved in cancer growth, invasion, angiogenesis, anti-apoptosis and epithelial to mesenchymal transition[45]. Two monoclonal antibodies, rilotumumab (an anti-HGF antibody) and onartuzumab (an anti c-MET antibody) were tested. In a phase I b/II study, rilotumumab was effective and it improved PFS[46]. Based on these data, the phase III RILOMET-1 trial, conducted on selected c-MET amplified patients, evaluated OS and ORR in the experimental arm with rilotumumab plus ECX compared to control arm with placebo plus ECX. The trial results were negative, and demonstrated that rilotumumab does not improve survival[47]. A similar phase III study called RILOMET-2 is ongoing for Asian patients in the same setting[48].

Onartuzumab, a monoclonal antibody directed to c-MET, was tested in MET-Gastric study, in which patients were randomized to receive FOLFOX alone or in combination with onartuzumab. Once again, results were negative (OS: 11.0 mo in the experimental arm vs 11.3 mo in the control arm, HR = 0.82, P = 0.24)[49]. Recently results on a specific MET kinase inhibitor have been presented at ASCO 2016[50]. For the first time AMG337 was tested, in a phase I study, in humans with solid tumors: 51 patients were treated and among them 10 had MET-amplified gastrointestinal cancers: 4 partial responses and 1 complete response were observed. At the end of the study a maximum tolerated dose of 300 mg was reached. Although an expansion phase on MET-amplified patients was on the way, it was early interrupted for excess of toxicity. Despite these negative results, the interest on c-MET as a potential molecular target for novel therapies has not vanished, since better molecular selection of the patients and optimal combination/drugs may finally achieve the expected results.

VEGF and VEGFR-2

Another frequently amplified gene in CIN subtype is VEGF, a mediator of angiogenesis that is essential for cancer growth and metastasis as it ensures oxygen and nutrients supply to proliferating cancer cells[51]. Bevacizumab, a monoclonal antibody that targets VEGF, was tested in the AVAGAST trial. This study did not meet its primary endpoint of improved OS (median OS 12.1 mo vs 10.1 mo, HR 0.87 95%CI: 0.73-1.03, P = 0.1), but improvements in median PFS and tumor response rate were reported[52]. Similarly, the AVATAR trial showed no survival benefit with antiangiogenic therapy added to cisplatin and capecitabine-based regimens (HR 1.1)[53]. Although the addition of bevacizumab to standard therapy showed disappointing results, antiangiogenic strategy was further investigated beyond first line treatment. Ramucirumab, a fully human monoclonal IgG directed against VEGFR-2, was evaluated both as single agent and in combination with chemotherapy[54,55]. In the REGARD trial, ramucirumab demonstrated a statistically significant improvement when compared to the best supportive care in pretreated GC patients with advanced disease (OS: 5.2 mo vs 3.8 mo respectively, HR = 0.776; P = 0.047)[54]. In the RAINBOW trial, patients were randomized to receive paclitaxel with or without ramucirumab. Median OS was 9.63 mo for the combination therapy and 7.36 mo for paclitaxel alone (HR = 0.807, 95%CI: 0.678-0.962; P = 0.017)[50]. Recently, a novel VEGFR-2 tyrosine kinase inhibitor, apatinib, was evaluated in Asian patients who had previously received 2 or 3 lines of chemotherapy[57]. Patients exposed to apatinib had an improved median OS (6.5 mo vs 4.7 mo; HR = 0.709; 95%CI: 0.537-0.937; P = 0.156) and median PFS (2.6 mo vs 1.8 mo; HR = 0.444; 95%CI: 0.331-0.595; P < 0.001) compared to patients who received placebo. Therefore, multitarget TKIs represent another potential approach to block angiogenesis by simultaneously targeting VEGFR and other signaling pathways. Notably, the role of antiangiogenic strategy seems to gain importance in subsequent lines of treatment, but its role in first-line therapy is still unclear. An ongoing randomized phase III trial is assessing the potential survival benefit of ramucirumab in combination with cisplatin and capecitabine given upfront[56].

GC WITH MICROSATELLITE INSTABILITY

According to the TCGA’s molecular classification, the enrichment for microsatellite instability (MSI) characterizes a distinct molecular subgroup of GC. MSI occurs in about 15%–30% of GCs, and more frequently correlates with intestinal histotype, location in the distal part of the stomach, female gender and older age at diagnosis[5,58,59].

MSI is a genetic alteration consisting of the expansion or contraction of regions of repetitive nucleotide sequences, called microsatellites. The alteration is triggered by a dysfunction of DNA mismatch repair (MMR) enzymes, caused by mutations in one of several different DNA mismatch repair genes (i.e., MLH1 or MSH2). In a single cell, bi-allelic inactivation of MMR genes causes an increased mutation rate (genomic instability) due to the failure of DNA mismatch repair that usually occurs during normal DNA synthesis[60].

Defective DNA mismatch repair is the hallmark of Lynch syndrome. Moreover, approximately 15% of sporadic colorectal cancers also displays MSI since both alleles of a MMR gene are inactivated[61]. Different MMR genes are probably involved in MSI-high (MSI-H) sporadic gastric cancer without MLH1 hypermethylation, which represents the main mechanism leading to MMR deficiency in MSI GC[62,63].

MSI-H colorectal cancer have better prognosis compared to MSI low, and should not receive adjuvant chemotherapy with fluoropyrimidine after resection for stage II disease[64]. In gastric cancer, 5-FU is frequently used and information about sensitivity to this agent may be very useful. A meta-analysis of Zhu et al[65] showed a 37% mortality risk reduction and improved median OS in patients with MSI-H compared to MSI-L(low) or microsatellite stable (MSS) GC patients. The relationship between MMRD, MSI and survival has been examined in patients with resectable GC randomized to surgery alone or perioperative chemotherapy within the MRC MAGIC...
trial. MSI and MLH1 deficiency was associated with a better outcome in patients treated with surgery alone while it had a negative prognostic effect in those treated with chemotherapy[62].

Despite MSI cases generally lack of targetable amplifications, mutation in PIK3CA, ERBB3, ERBB2 and EGFR are noted[59]; BRAF V600E mutations, commonly seen in MSI colorectal cancer, are absent in MSI GC[5]. However, the predictive role of these mutations in MSI GC population is uncertain. The combination of olaparib with paclitaxel as second-line therapy was found to be more active compared with paclitaxel alone in patients with metastatic or recurrent GC. Although the trial did not meet its primary endpoint (namely PFS), olaparib prolonged survival in patients with low levels of ataxia telangiectasia mutated, a key activator of DNA damage response[66]. A phase III trial in this setting is under way and detailed analysis in MSI GC could be attractive.

The hypothesis of an increased activity of immunotherapy in MSI non-colorectal cancer has recently generated interest. In fact, the increased number of somatic mutations may amplify the number of neoantigens, thus stimulating the immune system and conferring higher sensitivity to PD-1 blockade to tumor[67,68]. Interestingly, the tendency to have a lymphocytic infiltrate, observed in MSI tumors, likely reflects immune activation of T-cells directed against tumor-specific carboxy-terminal frameshift peptides that are associated with MSI[69]. In addition to that, genomic aberrations in tumor cells lead to aberrant PD-L1 expression, suggesting a predictive role for MSI.

MSI has already been reported as a strong predictive factor for the use of immune check-point inhibitors in the treatment of patients with colorectal cancer[70]. The immune-related objective response rate and immune-related 6-mo PFS rate were 40% and 78%, respectively, for patients with dMMR and 0% and 11% for those with MMR-proficient cancer, with a higher median PFS and survival in the cohort with dMMR colorectal cancers vs 2.2 and 5.0 mo, respectively, in the cohort with MMR-proficient tumors. Le et al[66] enrolled 41 consecutive patients (9 patients with MMR deficient solid tumors other than colorectal cancer, only 1 patient with GC) to explore the activity of PD-1 blockade according to MMR status in non colorectal cancer too. Although data are not ready for clinical application, 30% of GC have been shown to present with a burden of nonsynonymous mutations that may define who are the optimal candidates for immune check-point inhibitors treatment[71]. Of note, a phase 2 study of pembrolizumab in subjects with advanced gastric or gastroesophageal junction adenocarcinoma who progressed after first-line therapy with platinum and fluoropyrimidine is currently recruiting participants[72]. Muro et al[73] have recently reported the activity of pembrolizumab in GC in a phase I trial. The authors showed a decrease in tumor burden in 41% of the study patients. The ORR was 32% in Asian patients and 30% in non-Asian patients[73]. A phase 2 trial of nivolumab or nivolumab plus ipilimumab is recruiting patients to evaluate the response to checkpoint inhibitors in MSI-H gastrointestinal cancers[74]. Interestingly, a preventive vaccine, set-up using neopeptides frequently affecting MSI tumorigenesis, has been shown to delay the onset of dMMR tumors. It remains to be proven if vaccination against these neopeptides might be a promising approach for novel adjuvant treatment strategies in patients with MSI-H tumors[75].

### GC WITH GENOMIC STABILITY

GS GCs account for around 20% of all the tumors analyzed by the TCGA project. This subtype occurs with equal frequency in males and females. GS gastric tumors are enriched for the diffuse histological variant [58% according to Lauren’s classification] and for the poor cohesive variant (58% according to World Health Organization (WHO) classification). One quarter of GS GCs arise in the antrum, about 20% in the gastroesophageal junction/cardia, and approximately 15% in the gastric body/fundus. The principal somatic genomic alterations observed in GS gastric tumors involve CDH1, ARID1A and RHOA. In addition, a recurrent interchromosomal translocation (between CLDN18 and ARHGAP26) implicated in cell motility was found in GS gastric tumors[5].

### CDH1

The CDH1 gene is located on chromosome 16q22.1 and encodes E-cadherin, which belongs to the cadherin superfamily of calcium-dependent cell adhesion molecules. E-cadherin plays a well-documented role in the progression of epithelial cancers. Inactivating mutations in the CDH1 gene are frequently found in gastric cancer, especially in hereditary diffuse gastric cancer[76]. CDH1 promoter methylation is also frequently found in sporadic gastric cancers[77]. During epithelial tumorogenesis, the protein is downregulated and E-cadherin has been categorized as a tumor suppressor gene[78]. Li et al[79] reported that in diffuse-type GC, CDH1 mutation is associated with shortened patients survival, independently from disease stage. In the analysis of the TGCA Research Network CDH1 somatic mutations were enriched in the GS subtype (37% of cases). Therefore, the prognostic value of CDH1 as well as its potential as therapeutic target in gastric cancer has yet to be fully understood and explored.

### ARID1A

Inactivating mutations of ARID1A were found in GS gastric cancer, as in the EBV-subtype[5]. The ARID1A gene, located in chromosome 1p35.3, encodes adenine-thymine-rich interactive domain-containing protein 1A, which participates in chromatin remodeling, therefore is involved in regulating cellular processes including DNA repair, differentiation, and development[80]. As shown by Wang et al[81], loss of ARID1A expression was significantly correlated with tumor stage and grade; moreover, it was also significantly correlated with poor survival in GC patients. Restoring ARID1A expression in gastric cancer cells significantly inhibited cell proliferation and colony formation, whereas silencing ARID1A expression...
in gastric epithelial cell lines significantly enhanced cell growth rate\textsuperscript{81}.

**RHOA**

Rho belongs to the Ras-related family of small molecular weight GTP-binding proteins, and it works as a molecular switch between the GDP-bound inactive form and the GTP-bound active form\textsuperscript{82}. It regulates cytoskeletal organization, cell adhesion, intracellular membrane trafficking, gene transcription, apoptosis, and cell cycle progression\textsuperscript{83}; moreover, it activates STAT3 to promote tumorigenesis\textsuperscript{84}. RhoA plays a role in these processes through a variety of effectors including ROCK1, mDia and protein kinase N\textsuperscript{85}. mDia is involved in nucleation and polymerization of actin filaments, while ROCK intervenes in induction of actinomyosin bundles and contractility.

The balance between mDia and ROCK regulates cell morphogenesis, adhesion, and motility activities. In addition, the Rho–ROCK pathway is involved in Ras-mediated transformation, the amoeboid movement of tumor cells in the three-dimensional matrix, and transmigration of tumor cells through the mesothelial monolayer\textsuperscript{86}. According to the TCGA, RHOA mutations were clustered in two adjacent amino-terminal regions that are predicted to be at the interface of RHOA with ROCK1 and other effectors, leading to a modulation of signaling downstream of RHOA\textsuperscript{87}. Interestingly, diffuse-type GCs, characterized by malignant phenotype and stromal differentiation, frequently have gain-of-function mutations of RHOA\textsuperscript{87}.

The TCGA network discovered a recurrent inter-chromosomal translocation between claudin 18 (CLDN18) and Rho GTPase-activating protein 6 (ARHGAP26), resulting in the CLDN18-ARHGAP26 fusion gene, which primarily occurs in GS GC\textsuperscript{5}. ARHGAP26 (also known as GTPase Regulator Associated with Focal Adhesion Kinase, GRAF) is a GTPase-activating protein that facilitates conversion of RHO GTPases to the GDP state and has been implicated in enhancing cellular motility\textsuperscript{88}. CLDN18 is a component of the tight junction adhesion structures\textsuperscript{89}. Yao et al\textsuperscript{90} showed that expression of CLDN18-ARHGAP26 fusion gene in gastric epithelial cells resulted in epithelial–mesenchymal transition, which is indicative of cell transformation in cancer development. A recent trial tested IMAB362, a chimeric IgG1 antibody against CLDN18.2 showing clinical activity in patients with 2+/3+ immunostaining\textsuperscript{91}.

The CLDN18-ARHGAP fusions were mutually exclusive with RHOA mutations; within the GS subtype, 30% of cases had either RHOA or CLDN18-ARHGAP alterations\textsuperscript{92}.

Given the role of RHOA in cell motility, modulation of RHOA may contribute to the disparate growth patterns and lack of cellular cohesion that are hallmarks of diffuse tumors.

Rho/Rho-kinase inhibitors have been explored as putative therapeutic targets in various diseases, including cancers\textsuperscript{93}. The development of drugs that inhibit Rho GTPase signaling would be of great potential in this setting.

**Other notable patterns**

The GS subtype exhibited elevated expression of cell adhesion pathways, including the B1/B3 integrins, syndecan-1-mediated signaling, and angiogenesis-related pathways. Also in the GS subtype, hierarchical clustering of samples and pathways revealed several notable patterns, including elevated expression of mitotic network components such as AURKA/B and E2F, targets of MYC activation, FOXM1 and PLK1 signaling and DNA damage response pathways\textsuperscript{94}. Specific inhibitors of AURKA are currently under investigation in phase I/II clinical trials in advanced GC\textsuperscript{95}. PLKs, mitotic kinases of the polo family, play a pivotal role in the normal cell cycle, and their overexpression is involved in the pathogenesis of multiple human cancers\textsuperscript{96}. PLK1 is overexpressed in approximately 80% of human tumors, including gastric cancer, and it is associated with poor prognosis\textsuperscript{97}. Currently, inhibitors of PLK1 are being developed\textsuperscript{98}. In a phase I trials enrolling patients with advanced solid cancers, including gastric cancer, volasertib, a potent and selective PLK inhibitor that induces mitotic arrest and apoptosis, demonstrated anti-cancer activity with a manageable safety profile\textsuperscript{99}.

**EBV ASSOCIATED GC**

Latent EBV infection is associated with about 10% of GCs, as demonstrated by in situ hybridization EBV encoded miRNA detection, by whole genome sequencing or by PCR EBV genome detection\textsuperscript{100}.

EBV associated GC has been related to different epidemiological and clinico-pathological features. In a meta-analysis of 39 case-control studies, Bae et al\textsuperscript{97} investigated the strength of association between EBV infection and GC risk, and showed a 10 fold increase (95%CI: 5.89-17.29). It was also reported that there is a higher risk of EBV associated GC in Far East Asia if compared to Europe\textsuperscript{101}.

In a meta-analysis of 70 studies the pooled prevalence of EBV-positive GC resulted 8.7% (95%CI: 7.5%-10.0%) with similar distributions across the three analyzed geographic regions (America, Asia and Europe). Moreover, a two-fold difference in male/female ratio favored men as to prevalence of EBV positive GC. The antral location was less frequently associated with EBV infection when compared to other types. In contrast, there was no statistically significant difference in the proportion of EBV-positive disease between intestinal (9.5%; 95%CI: 7.2%-12.5%) and diffuse (7.6%; 95%CI: 5.7%-10.3%) histology\textsuperscript{98}.

In addition, EBV-positive GC was more prevalent in younger patients compared to older subjects\textsuperscript{99}.

As to possible therapeutic approaches, Kim et al\textsuperscript{100} observed that EBV infected GC patients had a higher rate of alteration in pathways related to immune response which may also be related to a more favorable prognosis.
in these patients. According to TCGA, PD-L1 gene was frequently amplified in EBV-positive GC, adding proofs to the hypothesis of higher immunogenicity of this class of GC. Based on the evidence that 15% of EBV positive GC harbored amplification of chromosomal region 9p24.1, the locus of PD-L1 and PD-L2, potential role of PD-L1 expression in EBV-positive GC was investigated in a study. In EBV-associated GC, PD-L1 expression was present in 50% (16/32) and 94% (30/32) of tumor and immune cells, respectively. In contrast, EBV-negative GC showed a lower PD-L1 expression (10% and 39% of tumor and immune cells, respectively, P < 0.001), thus providing a further rationale for testing PD-1 expression in this GC subtype to potentially identify a predictive response factor for immunomodulatory therapeutic strategies.

Besides PD-L1 and PD-L2 expression, PIK3CA mutations, DNA hypermethylation, and JAK2 mutations are also present. In a large retrospective study, 855 GC specimens were analyzed to verify protein expression levels and prognostic values of PIK3CA, JAK2, PD-L1 and PD-L2. Only 59 samples were found to be EBV positive. PIK3CA and PD-L2 were more highly expressed in EBV positive GC than in negative ones, but no prognostic value of PIK3CA, JAK2, PD-L1 or PD-L2 was found. No differences in JAK2, PD-L1 or PD-L2 expression were seen between EBV positive and negative cases. Moreover, the expression of PIK3CA, JAK2, PD-L1 or PD-L2 was not significantly associated with any clinicopathological feature, maybe due to the small number of EBV-associated GC cases, and the prognostic value of these mutations remains uncertain.

THE ACRG CLASSIFICATION

The ACRG proposed a different molecular classification for gastric cancer in 2015. This classification has some overlapping features with the one proposed by TCGA even though some differences can be highlighted. The clustering process included a first subdivision into MSI (22.7%, better prognosis, mainly intestinal type) and EMT tumours (15.3%, worse prognosis mainly diffused type) with two exclusive gene expression profiles, the first characterized by the loss of function of genes involved in the MMR and the second by alterations in cell adhesion, angiogenesis, and motility. Notably, the MSI subtype was associated with a hypermutation in genes such as: KRAS (23.3%), PI3K-PTEN-mTOR pathway (42%), ALK (16.3%) ARID1A (44.2%), ERBB2 (16.3%) and ERBB3 (14%). The remaining tumours were further divided into MSS/TP53 (26.3%, P53 function intact) and MSS/TP53 (35.7%, loss of oncopressor function). In terms of survival, the MSI subtype showed the best overall prognosis, followed by MSS/TP53, MSS/TP53 and MSS/EMT. The MSI/TP53 subtype was more frequently associated with EBV infection if compared to the other groups and showed an active TP53 pathway and a higher prevalence (compared to MSI/TP53 of APC, ARID1A, KRAS, PI3KCA, and SMAD4 mutations. Finally, the MSI/TP53 subtype showed the highest prevalence of TP53 mutations, relevant copy number variations (CNVs), a greater aneuploidy and recurrent focal amplifications in MD2, ROBO2, GATA6, MYC, ERBB2, EGFR, CCNE1 and CCN1. These latter two amplifications were mutually exclusive, so they could be considered driver alterations.

A comparison of the ACRG categories with the TCGA subtypes showed similarities in the tumors with MSI, while GS was approximated to MSS/EMT, EBV to MSS/TP53, and CIN to MSS/TP53. Nevertheless, in the TCGA cohort the EBV positive cancers represented a separated subgroup (with a favourable phenotype), whereas in the ACRG classification EBV infection occurred more frequently in the MSS/TP53 subtype, without CNVs, hypermethylation or hypermutation. Moreover, PIK3CA and ARID1A mutations were more prevalent in EBV gastric cancers compared to MSS subtypes.

Although both the MSS/EMT and the GS molecular subgroups included tumors with a prevalent diffuse histology, the TCGA classification showed a lower percentage of Lauren’s diffuse subtype compared to the ACRG database (24% vs 45% respectively); additionally, CDH1 and RHOA mutations did not appear prevalent in the MSS/EMT subgroup, unlike the GS subtype. Finally, GS tumours were also present in the ACRG MSS/EMT, MSS/Tp53 + and MSS/tp53 molecular subgroups. All these findings showed that the GS and the MSS/EMT subgroups were not equivalent.

The comparison of the CIN TCGA subtype to ACRG MSS/TP53 subtype showed that the first is quite homogeneously distributed in the subtypes classified by ACRG.

Overall survival associations were weaker when using the TCGA genomic scheme in the ACRG cohort compared to the original prognosis trends: While the MSI subtype showed a better prognosis in both classifications, there were no differences in prognosis in CIN and GS subtypes when they were identified based on application of the TCGA classification on the ACRG patient population.

CONCLUSION

While the advent of novel molecular classifications has faded the "one size fit-all" era, a more profound understanding of the underpinning tumour biology has set the dawn of a more contemporary clinical approach called precision medicine. At present, the two aforementioned genomic classifications of GC represent the state-of-the-art achieved so far. Somehow it is possible to find an overlap between the TCGA and ACRG subtypes even though some difference can still be found. Emerging data clearly individuate a category of GC characterized by MSI that may benefit from immunotherapeutic approaches. For this subgroup, with good prognosis, the development of anti-PD-1/PD-L1 drugs could be the leading research avenue. High mutational burden is also a driving feature of EBV positive GC that could be targeted with immunotherapy as...
efficaciously as in MSI tumours.

It is also possible to clearly segregate another class of GC classified either as GS or MMS/EMT, in which the prevalent deregulation is represented by EMT pathway alterations. Development of inhibitors of HGF/c-Met pathway, Rho/Rho-kinase, AURKA/AURKB, PLK1 could be a strategy adopted in the near future.

The category corresponding to CIN, and partially to MSS/TP53, represents a cluster of GC with high CNV variation leading to deregulation of specific biological targets such as receptors and kinases. Since these driver alterations are mostly mutually exclusive, they could be easily targeted using specific monoclonal antibodies or TKIs. On the other side, tumour heterogeneity may limit the efficacy of targeted strategies through alternative targets such as receptors and kinases. Since these driver alterations are mostly mutually exclusive, they could be easily targeted using specific monoclonal antibodies or TKIs. On the other side, tumour heterogeneity may limit the efficacy of targeted strategies through alternative mechanisms of primary and acquired resistance.[39]

The overall landscape is complex and our knowledge on this topic is still just at the starting point and novel trials should be designed accordingly (Table 1).

### Table 1 Clinical outcomes of recent trials in gastric and esophagogastric adenocarcinomas

| Trial name | Phase of study | Line of treatment | Selected biomarker | Treatment arms | n | Primary endpoint | Outcomes |
|------------|----------------|-------------------|--------------------|----------------|----|------------------|----------|
| CIN        | II              | First             | HER2 expression/amplification | CF/CX + trastuzumab | 296 | OS | 15.8 mo vs 11.1 mo (HR = 0.74, P = 0.005) |
|            | III             | First             | HER2 expression/amplification | CapoOX + laptinib | 273 | OS | 12.2 mo vs 10.5 mo (HR = 0.91, P = 0.34) |
|            | III             | Second            | HER2 amplification by FISH | Paclitaxel + laptinib | 129 | OS | 11.0 mo vs 8.9 mo (HR = 0.84, P = 0.104) |
|            | III             | First             | HER2 expression/amplification | Pertuzumab + tPF Placebo + tPF | 117 | OS | Ongoing |
| EXPAND     | III             | First             | Unselected | CX + cetuximab | 44 | OS | 8.6 mo vs 7.9 mo (HR = 1.15, P = 0.86) |
| REAL-3     | III             | First             | Unselected | EOC + panitumumab | 278 | OS | 11.0 mo vs 8.6 mo (HR = 1.37, P = 0.013) |
| RILOMET -2| III             | First             | MET positive by IHC HER2 negative | ECX + rilotumumab | 305 | OS | 11.5 mo vs 9.6 mo (HR = 1.37, P = 0.016) |
| METGastra  | III             | First             | MET positive by IHC HER2 negative | mFOLFOX + ornatuzumab | 562 | OS | 6.8 mo vs 6.7 mo (HR = 0.90, P = 0.429) |
| AVAGAST    | III             | First             | Unselected | CX + bevacizumab | 387 | OS | 10.1 mo vs 12.1 mo (HR = 0.87, P = 0.1) |
| AVATAR     | III             | Unselected        | CX + bevacizumab | 102 | OS | 11.4 mo vs 10.5 mo (HR = 1.11, P = 0.55) |
| REGARD     | III             | Progression after TP | Unselected | BSC + ramucirumab | 117 | OS | 5.8 mo vs 5.2 mo (HR = 0.77, P = 0.047) |
| RAINBOW    | III             | Second            | Unselected | Paclitaxel + ramucirumab | 335 | OS | 7.4 mo vs 9.6 mo (HR = 0.80, P = 0.017) |
| Apatinib   | III             | Third or more     | Unselected | Placebo Apatinib | 91 | OS | 4.7 mo vs 6.5 mo (HR = 0.70, P = 0.015) |
| MSI        |                |                   |                   |                |    | Ongoing |
| NCT01063517 | II              | Second            | ATM expression | Paclitaxel + olaparib + Pembrolizumab | 62 | OS | 8.3 mo vs 13.1 mo (HR = 0.56, P = 0.01) |
| NCT02889496 | II              | Second            | Unselected | EOX + IMAR362 | 161 | OS | 8.7 mo vs 12.5 mo (HR = 0.5) |
| FAST       | II              | First             | CLDN18.2 | EOX + IMAR362 | 161 | OS | 5.7 mo vs 7.9 mo (HR = 0.5, P = 0.01) |

Most significant target-oriented phase II and phase III trials are presented. In the table are shown in order: name of the trial, phase of the study, line of treatment, biomarker selection, treatment arms, number of enrolled patients, primary endpoint and key outcome results. tPF: Trastuzumab + Platinum + fluorouracil; PF: Platinum + fluoropyrimidine; TAX: Taxane; CF: Cisplatin + fluorouracil; CX: Cisplatin + capecitabine; EOC (or ECX): Epirubicin + Fluorouracil + Cisplatin; BSC: Best supportive care; CIN: Chromosomal instability; GS: Genomical stability; MSI: Microsatellite instability.
tumour subtypes and setting apart patients with different diseases will represent the future of gastrointestinal oncology. The key landmark comprehensive efforts made by TCGA and ACRG have just paved the way for precision oncology.

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