Heterogeneity in Gastric Cancer: From Pure Morphology to Molecular Classifications

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Keywords

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Abstract

Gastric cancer (GC) represents a global health concern. Despite advances in prevention, diagnosis, and therapy, GC is still the third leading cause of cancer mortality worldwide, with more than 720,000 estimated deaths in 2012. Overall survival for advanced disease is about 1 year, a dismal prognosis that is partly due to the high levels of biological heterogeneity found in GC. Indeed, GC is a highly heterogeneous disease from morphological and molecular standpoints. The numerous histological and molecular classifications currently available reflect such heterogeneity. Although recent high-throughput studies cluster the molecular data obtained into subgroups with clinical relevance, we still need a practical, prognostic, and predictive classification system, integrating morphological and molecular features, towards the identification of novel therapeutic targets. It is noteworthy that GC heterogeneity encompasses not only interpatient variability (intertumour heterogeneity), but also variations within the same tumour (intratumour heterogeneity). The latter encompasses spatial heterogeneity (in different

tumour areas) and temporal heterogeneity (along progression from primary to recurrent and/or metastatic disease). In this review, we analyse the morphological, immunophenotypic, and molecular heterogeneity in GC as the basis for a better understanding of the disease, and discuss the practical implications for diagnostic pathology, prognostic evaluation, and precision therapy.

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Introduction

Gastric cancer (GC) constitutes an important health problem. It is the fifth most common cancer and the third leading cause of cancer mortality worldwide [1]. Despite a gradual decline of incidence and mortality in recent decades, and despite improvements in prevention, diagnosis, and therapeutic approaches, the burden remains high. Based on GLOBOCAN estimates, 952,000 GC cases were diagnosed in 2012, causing 723,000 deaths [1].

The high mortality of GC is mainly due to late diagnosis and poor response to the currently available therapeutic regimens. One of the reasons that may explain the poor clinical outcomes of GC therapy is the highly heterogeneous nature of this disease. Tumour heterogeneity can be

due to genetic alterations, epigenetic events, interactions between tumour cells and the microenvironment, and also interactions between the different tumour cell clones/populations within the tumour. Such heterogeneity involves geographical variations, macroscopic and microscopic features, as well as molecular alterations. Moreover, phenotypic and molecular diversity encompasses not only interpatient variations (intertumour heterogeneity), but also variations within the same tumour (intratumour heterogeneity; ITH). However, currently, few histopathological and/or molecular features are taken into account in the clinical management of GC patients. Adequate surgical resection, or endoscopic resection in selected early-stage tumours [2], are the mainstays of the curative approach. Fluoropyrimidine- and platinum-based chemotherapeutic regimens are recommended in the neoadjuvant or adjuvant setting, and as the first-line treatment in patients with advanced and unresectable GC. Overall, objective response rates to chemotherapy range from 20 to 40%, indicating variable clinical responses, probably caused by underlying biological heterogeneity [3]. In addition to conventional chemotherapeutic regimens, targeted therapies, consisting of small molecules or monoclonal antibodies able to disrupt specific oncogenic pathways, have recently emerged as new therapeutic strategies. Based on phase III clinical trials, trastuzumab (anti-HER2; human epidermal growth factor receptor 2) [4] and ramucirumab (anti-VEGFR2; vascular endothelial growth factor receptor 2) [5, 6] monoclonal antibodies have recently been approved as clinically validated molecular targeted therapies in the treatment of advanced/metastatic GC. However, these therapies offer only a limited survival advantage of a few months (1.5–2.2 months) [4–6] and, to date, with the exception of HER2, there are no predictive biomarkers of tumour response to targeted therapies in GC [7]. Other biological agents (e.g., cetuximab and panitumumab, anti-EGFR monoclonal antibodies) have failed to demonstrate a survival benefit in GC [8, 9]. Very recently, cancer immunotherapies, able to impair the cancer evasion from immune surveillance, have been explored. Phase II/III clinical trials with antibodies directed against CTLA-4 and PD-1/PD-L1 immune inhibitory checkpoints are currently ongoing in GC and are new attractive therapeutic strategies for GC patients [10–13].

Overall, more effective treatment options are urgently required for improving GC patient outcome. Since tumour plasticity and, both inter- and intratumour heterogeneity are reported as putative contributors to treatment failure, it is pivotal to study/understand GC heterogeneity in order to develop more effective therapeutic regimens

that are able to target the heterogeneous tumour cell populations. Therefore, in this review article, we will discuss the inter- and intratumour heterogeneity of GC, from morphological and molecular standpoints, focusing on diagnostic, prognostic, and therapeutic implications.

Morphological Heterogeneity of GC

GC is very heterogeneous from the morphological standpoint. The large number of histopathological classifications proposed over the years testifies to the great variability of GC morphology. Moreover, it is worth pointing out that morphological ITH, i.e., the coexistence of different morphological components within the same tumour, is frequent, adding complexity to histological classifications and raising concerns about accuracy and reproducibility. Some of the histological classification schemes that have been proposed are those of Laurén (1965) [14], Nakamura (1968) [15], Ming (1977) [16], Goseki (1992) [17], Carneiro (1995) [18], Solcia (2009) [19], the World Health Organization (WHO; 2010) [20], and the Japanese Gastric Cancer Association (2011) [21] (Table 1). Figure 1 shows the variable histological patterns of GC, depicting the intertumour morphological heterogeneity.

Despite dating back to 1965, the Laurén classification [14], still remains widely accepted and used. It is based on a dichotomous scheme that distinguishes 2 main types: intestinal GC, forming tubular or papillary structures, and diffuse GC, characterised by poorly cohesive and infiltrative tumour cells that may or may not have a signet ring cell (SRC) morphology. The group of GCs which do not fit within these 2 major types encompasses mixed and indeterminate subtypes. Intestinal and diffuse GCs are clinically relevant subtypes, exhibiting distinct epidemiological profiles, histogenesis, clinicopathological and molecular features, as well as biological behaviour.

Intestinal GC most commonly occurs in elderly patients, mainly males, whereas diffuse GC is more common in young patients, mainly females. Both subtypes share environmental risk factors (e.g., *Helicobacter pylori* and Epstein-Barr virus infection, dietary habits, smoking); however, the diffuse GC pathogenesis is less well understood and presents a hereditary component. As a result, intestinal GC is more common in high-risk regions and is steadily decreasing in incidence, whereas diffuse GC is more common in low-risk areas and has a quite stable incidence [22, 23]. Moreover, cohesive versus non-cohesive morphological features probably correlate with

Table 1. Heterogeneity of histopathological classification systems in gastric cancer

Laurén [14], 1965	Carneiro [18], 1995	WHO [20], 2010	Japanese classification [21], 2011	Nakamura [15], 1968	Goseki [17], 1992	Solcia [19], 2009
Intestinal	Glandular	Papillary Tubular	Papillary Tubular 1 (well differentiated) Tubular 2 (moderately differentiated)	Differentiated type	I. Good tubular differentiation, mucin poor	Cohesive, ordinary subtype Cohesive, tubular subtype Cohesive, ordinary subtype
		Mucinous	Mucinous	Undifferentiated type	II. Good tubular differentiation, mucin rich	Mucinous, muconodular subtype Mucinous, infiltrative subtype
Diffuse	Isolated cell	Poorly cohesive, SRC phenotype	SRC carcinoma	Undifferentiated type	IV. Poor tubular differentiation, mucin rich	Diffuse, ordinary subtype
		Poorly cohesive, other cell types	Poorly differentiated, non-solid type		III. Poor tubular differentiation, mucin poor	Diffuse, low-grade desmoplastic subtype
Mixed	Mixed	Mixed				
Indeterminate	Solid	Undifferentiated	Poorly differentiated, solid type	Undifferentiated type	III. Poor tubular differentiation, mucin poor	Anaplastic
	Rare variants	Rare variants				High lymphoid response
SRC, signet ring cell.						

the different tumour spreading patterns: while intestinal GC tends to metastasize hematogenously to the liver, diffuse GC usually disseminates through peritoneal surfaces [20]. Finally, based on the original report by Laurén et al. [14] and more recent studies [24–26], diffuse GC appears to behave more aggressively than intestinal GC and has been identified as an independent poor prognostic factor.

In 1995, Carneiro et al. [18] further elucidated the biological features and prognostic significance of mixed GC. Mixed GC, defined by a dual pattern of differentiation – glandular/solid (intestinal) and isolated-cell carcinoma (diffuse) – carried, in a multivariate analysis, significantly worse prognosis than the 2 main types of the Laurén classification [18, 27]. As further proof, several authors demonstrated that mixed GCs display more aggressive features than “pure” intestinal and diffuse GCs, including larger tumour size, deeper invasion, lymphatic invasion, and lymph node metastases [27–31]. Interestingly, mixed GC shows a dual metastatic pattern (hematogenous metastases and peritoneal dissemination with lymph node metastases), suggesting a cumulative effect of the adverse behaviours of intestinal and diffuse-type GC [32]. Therefore, the recognition and report of this mixed subtype is essential and is currently included in the 2010 WHO classification [20].

The WHO classification [20] is based on the recognition of specific histological patterns, also observed in other segments of the gastrointestinal tract, providing a unifying approach. However, this classification does not show significant relationships with epidemiological, clinicopathological, prognostic, or molecular characteristics. The WHO classification describes tubular and papillary adenocarcinomas (corresponding to the Laurén intestinal subtype), mucinous adenocarcinoma, poorly cohesive carcinoma (corresponding to the Laurén diffuse subtype), mixed carcinoma, and other rare histological variants, with the latter accounting for a small percentage of cases (about 5%). The WHO bluebook dedicates an entire chapter to hereditary diffuse gastric cancer (HDGC), the first hereditary syndrome identified in the stomach. HDGC is an autosomal dominant cancer-susceptibility syndrome that accounts for 1–3% of familial GCs and is characterised by a diffuse morphology and specific biological behaviour [33]. Heterozygous germline mutations have been recurrently found in the E-cadherin gene (*CDH1*, in 28–40% of cases) [34, 35] and α -E-catenin gene (*CTNNA1*) [35, 36], highlighting the inactivation of these two genes as the cause of HDGC. Recently, potentially deleterious mutations have been identified in the *BRCA2*, *STK11*, *SDHB*, *PRSS1*, *ATM*, *MSR1*, and *PALB2*

Fig. 1. Morphological heterogeneity in GC. Several histological patterns are represented according to the WHO classification. **a** Tubular adenocarcinoma, well differentiated. HE. Original magnification $\times 100$. **b** Papillary adenocarcinoma, well differentiated. HE. Original magnification $\times 40$. **c** Poorly cohesive carcinoma, composed of SRCs (left side) and poorly cohesive, pleomorphic cells (right side). HE. Original magnification $\times 400$. **d** Mixed carcinoma, displaying glandular/intestinal (left side) and poorly cohesive/diffuse elements (right side). HE. Original magnification $\times 200$. **e** Mucinous adenocarcinoma, with extracellular mucinous pools. HE. Original magnification $\times 40$. **f** Undifferentiated carcinoma. HE. Original magnification $\times 100$. **g** Hepatoid carcinoma, a rare histological variant characterised by large and polygonal cells with an abundant cytoplasm, resembling hepatocellular carcinoma. HE. Original magnification $\times 200$. Intracytoplasmic hyaline and periodic acid-Schiff-positive, diastase-resistant (PAS-D) globules can be observed ($\times 400$; arrowheads and **inset** left), that display α -feto-protein (α FP) immunoreactivity ($\times 400$; α FP IHC, **inset** right). **h** GC with lymphoid stroma, a rare histological variant in which the tumour cells are widely separated by an abundant lymphoid stroma. HE. Original magnification $\times 100$.

genes in HDGC families [35], albeit in a very small fraction of cases, and still with uncertain significance. The study of prophylactic gastrectomy specimens from asymptomatic *CDH1* mutation carriers led to the identification, in a very high proportion of cases, of multiple intramucosal cancer foci (pT1a), composed of bona fide SRCs, which are smaller at the neck zone and progres-

sively enlarge towards the surface of gastric mucosa [20, 37]. Precursor intraepithelial lesions (pTis), which are rare and hard to identify, encompass in situ SRC carcinoma and a pagetoid spread of SRCs [20, 38]. In a recent study [39], we described the variable morphological spectrum of precursor, early, and advanced lesions in HDGC. We found that early lesions (pTis and pT1a) display an

“indolent phenotype,” composed of typical SRCs, while advanced HDGC (pT > 1) is characterised by an “aggressive phenotype,” with a mixture of SRCs and pleomorphic, bizarre, and diffusely infiltrative cells. Moreover, the “aggressive phenotype” was statistically associated with the Ki-67 high-proliferation index and p53 expression. Hence, the heterogeneous nature of GC in the hereditary setting may help to define prognostic markers of progression from indolent to widely invasive carcinoma.

Among the other histopathological classification systems, those proposed by Goseki et al. [40, 41] and Solcia et al. [42] are important predictors of outcome. The Goseki histological grading system [17] identifies four categories combining the degree of tubular/glandular differentiation and the amount of mucus in the cytoplasm. More recently, Solcia et al. [19] proposed a 3-tiered prognostic classification based on histological, clinicopathological, and molecular parameters (Table 1).

In addition to classifications based on tumour morphology, GC can also be classified by immunohistochemistry (IHC) on the basis of lineage-differentiation markers. MUC5AC and TTF1 are markers of surface gastric epithelium, MUC6 and TTF2 represent markers of mucous neck cells or pyloric glands, while MUC2, CD10, and CDX2 are intestinal differentiation biomarkers. According to their expression, four GC immunophenotypes have been recognised: the intestinal (expressing MUC2, CD10, CDX2), gastric (expressing MUC5AC/TTF1 and/or MUC6/TTF2), hybrid (expressing intestinal and gastric markers), and null phenotype [43–46]. These phenotypes have also been recognised in GC precursor lesions [47–49]. Several authors have found differential genetic and epigenetic alterations in gastric versus intestinal phenotypes [50, 51], although the association with specific clinicopathological features and the prognosis is still conflicting [45, 51]. During GC progression, a phenotypic switch may occur from gastric to intestinal differentiation [45]; moreover, some authors hypothesise that GC with a gastric phenotype may also progress to poorly differentiated GC [44, 47].

Moving towards Molecular Subtyping and Precision Medicine

A detailed characterisation of the GC molecular landscape is pivotal for the understanding of the biological behaviour of the disease, the identification of new molecular markers and, ultimately, for the improvement of diagnostic and therapeutic strategies. Gene mutations,

somatic copy number alterations, epigenetic changes, transcriptional changes, etc., have been studied over the years [52], revealing the extraordinary molecular heterogeneity of GC. Indeed, there is an urgent need to decipher such complex heterogeneity into clinical and biological homogenous subgroups.

With the recent advent of next-generation sequencing, several groups [3, 53–55] have analysed GC molecular alterations at high resolution, using various high-throughput platforms. These studies attempted to achieve an integrated molecular classification scheme, clustering the comprehensive molecular data obtained into subgroups with different molecular signatures and clinical phenotypes (Table 2).

Transcriptomic technologies have also been used to identify gene expression signatures with clinical relevance. A study by Tan et al. [3] started from GC cell lines to identify two “intrinsic” GC subtypes based on gene expressions (G-INT and G-DIF) that, when applied in GC samples, correlated with the Laurén classification, survival, and response to chemotherapy. These gene expression signatures were further analysed in 248 GC specimens by Lei et al. [53] (Singapore-Duke group), leading to the identification of three subtypes that show differences in molecular/genomic features, morphology, “carcinogenic” pathways, and response to therapy. These groups are: (1) the mesenchymal subtype, enriched with diffuse GCs and characterised by cancer stem cell and epithelial-to-mesenchymal transition (EMT) properties; (2) the proliferative subtype, with high levels of *TP53* mutations, genomic instability, and activation of oncogenic pathways; and (3) the metabolic subtype, overexpressing genes that are normally expressed in gastric mucosa. Proliferative and metabolic subtypes frequently correspond to the Laurén intestinal type. More recently, Das et al. [56] confirmed similar results using NanoString nCounter technology in 54 formalin-fixed and paraffin-embedded GC tissues. Their study further characterised the expression profiles of mesenchymal, proliferative, and metabolic subtypes and identified new biomarkers of response to mTOR inhibitors (RAD001), namely the overexpression of *MMP9* and *BRCA2*.

The landmark study of GC molecular-based stratification was carried out by The Cancer Genome Atlas (TCGA) research network [54], which proposed a four-tiered molecular classification that identifies: (1) Epstein-Barr virus-positive (EBV+) GC, characterised by recurrent *PIK3CA* mutations, frequent *JAK2* and *PD-L1* amplification, and a high level of DNA hypermethylation, as previously reported [57]; (2) GC with microsatellite in-

Table 2. Molecular classifications of GC and their therapeutic implications

Tan [3], 2011 (<i>n</i> = 270)			G-DIF (44%) Worst prognosis Cisplatin sensitive Diffuse GC	G-INT (56%) Best prognosis 5-FU and oxaliplatin sensitive Intestinal GC	
Lei [53], 2013 (<i>n</i> = 248)			Mesenchymal Low <i>TP53</i> mutations Low E-cadherin mRNA CSC/EMT properties mTOR inhibitors Diffuse GC	Proliferative High <i>TP53</i> mutations Genomic instability Oncogene amplification ¹ DNA hypomethylation Intestinal GC (intestinal phenotype)	Metabolic Low <i>TP53</i> mutations Normal gastric mucosa gene expression 5-FU sensitive Intestinal GC (gastric phenotype)
Bass [54], 2014; TCGA (<i>n</i> = 295)	EBV (9%)	MSI (22%)	GS (20%)	CIN (50%)	
	EBV-CIMP <i>CDKN2A</i> silencing <i>PIK3A</i> mutations <i>PD-L1/2</i> amplification <i>JAK2</i> amplification	Gastric-CIMP <i>MLH1</i> silencing <i>PIK3A</i> mutations <i>HER2/3</i> mutations <i>EGFR</i> mutations	<i>CDH1</i> mutations <i>RHOA</i> mutations <i>CLDN18-ARHGAP</i> fusion (<i>RhoA</i> -GTPase) Diffuse GC	High <i>TP53</i> mutations <i>TKR</i> -RAS amplification Amplification of cell-cycle mediators Intestinal GC	
Cristescu [55], 2015; ACRG (<i>n</i> = 251)		MSI (23%)	MSS/EMT (15%)	MSS/TP53– (inactive) (36%)	MSS/TP53+ (active) (26%)
		<i>MLH1</i> loss Hypermutation (<i>KRAS</i> , <i>ARID1A</i> , <i>PIK3A</i>)	<i>CDH1</i> loss	High <i>TP53</i> mutations Genomic instability Oncogene amplification ²	
	EBV+ cases included in MSS/TP53+	Best prognosis Intestinal GC	Worst prognosis Diffuse GC	Intermediate prognosis Intestinal GC	Intermediate prognosis Intestinal GC

GC, gastric cancer; TCGA, The Cancer Genome Atlas; ACRG, Asian Cancer Research Group; 5-FU, fluorouracil; CSC, cancer stem cell; EMT, epithelial-to-mesenchymal transition; EBV, Epstein-Barr virus; CIMP, CpG island methylation phenotype; MSI, microsatellite instability; GS, genomically stable; CIN, chromosomal instability; TKR, tyrosine kinase receptors; MSS, microsatellite stable.

¹ *ERBB2*, *CCNE1*, *MYC*, and *KRAS*.

² *ERBB2*, *EGFR*, *CCNE1*, *CCND1*, *MDM2*, *ROBO2*, *GATA6*, and *MYC*.

stability (MSI-high) characterised by DNA hypermethylation and *MLH1* silencing; (3) genomically stable GC, associated with a diffuse morphology and recurrent *CDH1* and *RHOA* events, as confirmed by previous studies [58–60]; and (4) GC with chromosomal instability (CIN), exhibiting intestinal morphology, a high number of *TP53* mutations, and amplifications of tyrosine kinase receptors (TKR).

EBV+ and MSI-high GCs are of particular interest. Morphologically, they are associated with prominent immune infiltrate [19] and frequently display the features of GC with lymphoid stroma [61–65]. There is growing evidence for the possibility of using PD-1/PD-L1 immune checkpoint inhibitors in these two molecular subtypes of GC, based on the following evidence: *PD-L1* amplification is frequent in EBV+ GC [54], and a recent study on colorectal, endometrial, small bowel, and gastric carcinoma cancer proved that MSI-high tumours treated with pembrolizumab (an anti-PD-1 monoclonal antibody) are associated with a higher rate of response and disease control compared to microsatellite stable (MSS) tumours

(ClinicalTrials.gov identifier: NCT01876511) [66]. Phase II clinical trials correlating response to pembrolizumab and molecular subtypes are currently ongoing in advanced GC (ClinicalTrials.gov identifier: NCT02589496). Several studies have demonstrated that PD-L1 immunorexpression is heterogeneous in GC, as in other tumour models. PD-L1 overexpression is associated with high densities of CD3+ and CD8+ tumour-infiltrating lymphocytes [67–69], GC with lymphoid stroma morphology [70], and both an EBV+ and MSI-high molecular status [69, 71–74]. However, the correlation with *PD-L1* amplification, prognosis, and response to targeted immunotherapies is still debated and deserves further study.

Finally, The Asian Cancer Research Group (ACRG) described four molecular subtypes with distinct prognostic impacts [55]: (1) MSI-high tumours, with an intestinal morphology and the best prognosis as previously described [75]; (2) MSS/EMT GC, with a diffuse morphology and the worst prognosis; and (3 and 4) MSS adenocarcinomas, with no EMT signature, either *TP53*-active (MSS/TP53+) or inactive (MSS/TP53–), and with an in-

intermediate prognosis. The MSS/TP53- subtype (which roughly corresponds to the proliferative and CIN subtypes) is frequent (36–50% of GCs) and harbours genomic amplifications of TKR and/or RAS, which are in use or potential therapeutic targets. In keeping with a previous study by Deng et al. [76], the ACRG found that TKR and RAS amplifications tended to be mutually exclusive, emphasising GC intertumour heterogeneity and the importance of investigating molecular alterations for targeted therapy.

As shown in Table 2, the three molecular classifications overlap only partially, highlighting the need for a consensual classification that may serve as a roadmap for patient stratification for prognostic evaluation and targeted therapy, in the era of precision medicine.

The tumour morphology may provide further insights into tumour biology and should be used as a frame for the identification of clinically relevant subgroups, as the backbone for building algorithms for directed and cost-effective molecular characterisation of GC. However, few correlation studies of morphological classification and molecular characteristics have been carried out [77, 78] and pathologists should be aware of their pivotal role in this field. In-depth studies combining molecular features with histopathological and IHC profiles may reveal interesting associations and are currently feasible, thanks to new molecular profiling technologies, generating accurate molecular information from formalin-fixed and paraffin-embedded tissues [56, 79]. On this note, Setia et al. [80] have recently proposed a practical algorithm based on IHC and in situ hybridisation techniques currently available in routine diagnostic practice. In their study, the authors accomplished to translate different molecular subgroups into specific immunophenotypes with prognostic and predictive significance.

Adding Further Complexity: ITH

The phenotypic and molecular heterogeneity of GC encompasses not only intertumour heterogeneity, as discussed above, but also ITH. ITH has been described as a new hallmark of cancer, which enables tumour cells to self-organise, invade, metastasise, and develop drug-resistance, through mechanisms of Darwinian selection and clonal evolution [81, 82]. ITH includes variability of different clones within the same tumour (spatial heterogeneity) [83], between primary tumour and recurrent disease (temporal heterogeneity), and between primary tumour and different metastatic sites. Such a degree of

ITH is predicted to have important implications in tumour diagnosis/classification, prognosis, and therapeutic resistance [84]. Assuming that every tumour is a heterogeneous entity, the collection of a limited number of biopsy samples raises important issues regarding the representativeness of the different populations in the overall tumour. This common lack of adequate sampling affects the correct evaluation of predictive biomarkers (e.g., HER2 expression) and even the histological diagnosis [85].

Mixed GC is the prototype of morphological ITH, as it is composed, by definition, of different histological patterns within the same tumour [20]. Glandular/intestinal and isolated-cell/diffuse components considerably vary in percentage, as well as in distribution, since the two components may be intermingled, adjacent, or completely separated [18, 86]. The recognition of this morphological entity is important, as it carries a bad prognosis compared to intestinal and diffuse GC (see Morphological Heterogeneity of GC, above). However, previous studies have shown that preoperative endoscopic biopsies have a limited value in predicting the presence of both components [29, 31, 87]. This is a clear example of how ITH may limit the correct pathological diagnosis and patient prognostic evaluation. The characterisation of molecular alterations underlying the two histotypes in mixed GC is of great interest and several authors have addressed this question. Machado et al. [88] demonstrated that inactivating E-cadherin somatic mutations were almost restricted to the diffuse component of mixed GC, providing a genetic basis for the phenotypic divergence of this type of GC. The finding that IHC expression of adhesion molecules (E-cadherin, α -catenin, β -catenin, γ -catenin) is more frequently preserved in the intestinal than diffuse component [30, 89] supports this hypothesis. Interestingly, some authors disclosed that the two components of mixed GC display similar DNA copy changes and CpG island hypermethylation status [90, 91]. Particularly, the two components of mixed GC have a higher number of methylated genes than “pure” diffuse and intestinal GC [91]. All these findings are in favour of a clonal origin of mixed GC and suggest that the diffuse component may emerge during the progression of some GCs, due to the occurrence of E-cadherin somatic mutations. Further studies are still required for a better understanding of other molecular events underlying the dual phenotypic differentiation of mixed GC.

HER2 is a transmembrane TKR of the epidermal growth factor receptor (EGFR) superfamily that regulates cell proliferation, survival, differentiation, migration,

Fig. 2. Spatial ITH of HER2 expression in GC. Both images represent well-differentiated tubular adenocarcinoma, with variable HER2 immunoreactivity between the tumour clones, from strong and complete membranous staining to weak and incomplete membranous staining. HER2 IHC, low magnification (**a**), $\times 100$ magnification (**inset**), and $\times 200$ magnification (**b**).

and other cancer-relevant cellular responses. In contrast to other HER family members, HER2 can dimerise and activate downstream effectors without ligand binding. Trastuzumab, an anti-HER2 monoclonal antibody, inhibits HER2 signalling, preventing the activation of the intracellular tyrosine kinase domain [92]. In 2010, the phase III ToGA (trastuzumab for GC) trial (ClinicalTrials.gov identifier: NCT01041404) demonstrated a significant improvement in response rate, progression-free survival, and overall survival with the addition of trastuzumab to chemotherapy compared with chemotherapy alone, in prestratified patients with HER2-positive GC/gastroesophageal junction cancer (GEJC) [4]. Since then, the use of trastuzumab with conventional chemotherapy has been approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) as first line therapy in advanced and metastatic HER2-positive GC and GEJC patients. An HER2-positive status, evaluated by IHC (3+) and/or fluorescence in situ hybridization (HER2+/FISH+), is predictive of the patient response to trastuzumab-based treatment. Overall, HER2 overexpression is observed in 10–38% of GCs [8, 77, 93–100]. Accurate testing for a HER2-positive status is, hence, mandatory to identify patients who will likely respond to this targeted treatment.

One major problem in this context is the heterogeneity of HER2 expression/amplification. Indeed, HER2 status varies across tumour locations (proximal GC and GEJC are more frequently positive [4, 77, 100]), histological subtypes (the HER2-positive rate is higher in intestinal

compared to diffuse GC [4, 101–111]), and molecular classifications (proliferative, CIN, MSS/TP53– and MSI GC are molecular clusters more frequently associated with *HER2* amplification [53–55, 80, 112]). Regarding mixed GC, some authors found that HER2 expression was restricted to the intestinal component [105], while others observed HER2 expression in both intestinal and diffuse components, in keeping with the hypothesis of clonal origin [107]. However, these studies evaluated only 4 and 2 mixed GCs, respectively, and further studies are necessary to clarify this issue.

Several authors have described ITH of HER2 expression in GC/GEJC. The extent of spatial heterogeneity of HER2 expression within primary tumours varies widely across studies, from 5 to 69% [94, 95, 106, 113–116]. These discrepancies can be explained by the lack of established criteria to evaluate and define HER2 ITH [117]. An interesting study indicated that HER2 expression is more frequent in the luminal tumour layers than deep ones and in the lateral regions compared to the central zone [118]. Moreover, HER2 ITH between primary tumours and metastatic sites, both lymph node [115, 119, 120] and hematogenous metastases [120–123], has been described, varying from 1 to 14%. Figure 2 shows the spatial ITH of HER2 expression in GC.

The heterogeneous expression of HER2 in GC/GEJC has several implications. First, it is one of the putative mechanisms that attenuate the response to trastuzumab-based targeted therapy, explaining the limited survival advantage described in the ToGA trial [4] and the devel-

opment of drug resistance [8]. Second, it can explain the controversial prognostic significance of HER2 status in GC/GEJC. In fact, while some studies showed that an HER2-positive status is associated with a worse prognosis and shorter survival [93, 98, 102, 110, 124, 125], others reported no prognostic value [103, 106, 107, 115, 126–129]. Third, HER2 heterogeneity constitutes a real challenge to render a correct and reproducible evaluation of the biomarker. For instance, because heterogeneity and incomplete membranous immunoreactivity of HER2 staining are frequent in GC/GEJC, pathologists had to develop a distinct HER2 scoring system, different from that used in breast cancer [94, 124, 130]. Another interesting problem is that of sampling errors, as HER2 expression evaluated in a portion of the tumour might not be representative of the HER2 status in the whole tumour. On this point, several authors have shown that the analysis of more than 1 tumour section increases the HER2-positive detection rate by 5–10% [111, 117, 131, 132]. With regard to HER2 status in matched biopsy and resection specimens, various authors reported a high concordance rate, ranging from 74 to 96% [111, 113, 115, 116, 133, 134]. Among the discordant cases, HER2 ITH may play an important role, highlighting the necessity of performing a relatively high number of endoscopic biopsies. Several studies, based on a comparative analysis between surgical resection specimens and endoscopic or virtual biopsies, recommended a minimum of 4–5 biopsies, preferentially from the proximal (oral part of the tumour), for reliable HER2 assessment [118, 135, 136].

To summarise, these findings show the importance of characterising HER2 ITH for guiding pathological analysis and clinical decisions, for example choosing one or more blocks with the greatest amount of intestinal and well-differentiated tumour for HER2 assessment, considering the repetition of HER2 testing in recurrent/metastatic disease, and performing multiple biopsies preferentially from the oral portion of the tumour when only biopsy samples are available.

Conclusions

GC is a heterogeneous disease from a histological and molecular standpoint. The current diagnostic reference, guiding clinical decisions, is still based on a histopathological evaluation. Advances in molecular characterisation have recently offered deeper insights into the heterogeneous nature of this disease and have highlighted the possibility of recognising molecular targets of therapy,

and creating more homogenous subgroups that may guide clinical decisions.

However, further studies are still needed, especially with respect to ITH. For instance, the understanding of molecular data in the frame of morphological/architectural information may be useful to better characterise spatial ITH, delineating the clonal evolution, the behaviour, and the relationship of different subclones, to better explain the prognosis and response to therapy and, ultimately, to build algorithms for precise and cost-effective molecular characterisation. Moreover, studying ITH, not only within primary tumours, but also in metastatic sites, will be pivotal for providing possible therapeutic solutions for advanced/metastatic disease.

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Disclosure Statement

The authors have no competing interests to declare.

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