Gastric Cancer: Applicability and Feasibility of Molecular and Histological Classification in Clinical Practice

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Abstract

Gastric cancer (GC) is the fifth most common type of cancer and the third leading cause of cancer-related deaths in the world. GC is a heterogeneous disease with diverse molecular and histological subtypes, which, may have different therapeutic implications. Using sophisticated molecular technologies and analyses, 3 separate groups recently provided genetic and epigenetic molecular classifications of GC: Singapore-Duke, The Cancer Genome Atlas project (TCGA) and the Asian Cancer Research Group (ACRG). These molecular classifications are time-consuming, complex, and costly and require sophisticated molecular technologies, which, prevent their widespread availability and use in clinical practice. Therefore, several practical pathological classifications were developed using immunohistochemical stains, fluorescent in situ hybridization and/or polymerase chain reaction (PCR), which, approximate, albeit not perfectly, the molecular classifications of GC. These are simple algorithms, less expensive and easy to reproduce in any pathology laboratory. Both molecular and histological classifications should be used for choosing adequate therapy and stratification purposes in clinical trials. This is a review of current molecular and pathological classification of GC.

Keywords: *gastric cancer, pathology classification, molecular classification*

Introduction

Gastric cancer (GC) is a major global health problem and the third leading cause of cancer related deaths worldwide. The incidence is highest in Asia (with over half of

all GC cases globally diagnosed in East Asia), Eastern Europe, and South America with comparatively lower rates in Africa, North America, and Europe. The overall survival for resected gastric cancer patients is poor; for US population, 5-year survival is

64% (local), 28.2% (regional) and 5.3% (distant metastases), with overall 5-year survival of 32.4% (1).

The disappointing survival rates are due to the limited treatment options and tumor heterogeneity, especially at the molecular level, with limited targetable biomarkers. Surgery is the 1st line treatment for GC, producing an overall survival rate of 60 - 70% for early-stage disease. High GC incidence nations such as Japan and Korea now implement routine screening for early detection when the disease is highly curable. In many less developed countries, however, GC is mostly detected only in its advanced stages, precluding curative surgical resection, and necessitating systemic treatment. The low efficacy of current therapies results in advanced or metastatic GC having a low survival rate of 5–20%, and a particularly poor prognosis for peritoneal GC recurrence (2).

Pathological Classification of Gastric Cancer

Historically, GC was classified morphologically by Lauren in 1965 in 2 main categories:

intestinal type and diffuse type (Fig.1) (3). This classification is still widely accepted by pathologists and clinicians in contemporary practice. Intestinal and diffuse GC exhibits numerous differences in pathology, epidemiology and etiology. *Intestinal type* is more common in older males, frequently associated with *Helicobacter pylori* infection, which, leads to gastric atrophy, intestinal metaplasia, low-grade dysplasia, high-grade dysplasia, and intestinal type adenocarcinoma, in a stepwise progression, known as Correa cascade (4). *Diffuse type* tends to occur in younger patients, mostly females and may occur in familial clusters. Histologically, these tumors lack expression of adhesion molecules and typically show an infiltrative, poorly differentiated, poorly cohesive appearance. Somatic and germline mutations in a number of genes contribute to diffuse type of GC: E-cadherin (CDH1), TP53, RHOA, CTNN1A, and CMTM2. Approximately 1–3% of all GC are due to hereditary diffuse gastric cancer (HDGC), with 30-40% of those associated with germline mutations in CDH1 (5).

Fig. 1. Lauren Classification of Gastric Cancer (1965)

Table 1. Evolution of Histological Classification of Gastric Malignant Epithelial Tumors

Lauren 1965	WHO 2000	WHO 2010	WHO 2019
Intestinal	Papillary Tubular (grade 1-3)	Papillary Tubular, well and moderately differentiated	Papillary Tubular, well and moderately differentiated
Indeterminate	Mucinous	Tubular, poorly differentiated (solid)	Tubular, poorly differentiated (solid)
Indeterminate/ intestinal/diffuse		Mucinous	Mucinous
Diffuse	Diffuse (signet ring carcinoma)	Poorly cohesive carcinoma (including signet ring carcinoma and other variants)	Poorly cohesive carcinoma NOS Signet ring carcinoma
Mixed adenocarcinoma	Mixed adenocarcinoma	Mixed adenocarcinoma	Mixed adenocarcinoma
Not defined	Histological variants of carcinoma: Adenosquamous carcinoma Squamous cell carcinoma Undifferentiated carcinoma Small cell carcinoma Others Carcinoid	Histological variants of carcinoma: Adenosquamous carcinoma Squamous cell carcinoma Undifferentiated carcinoma Carcinoma with lymphoid stroma (medullary) Hepatoid carcinoma Others Neuroendocrine neoplasms: <i>Neuroendocrine tumors (NET)</i> NET G1 (carcinoid) NET G2 <i>Neuroendocrine carcinoma (NEC)</i> Large cell Small cell Mixed adeno-neuroendocrine carcinoma (MANEC) ECL and serotonin producing NET Gastrin producing NET (gastrinoma)	Adenocarcinoma: Carcinoma with lymphoid stroma Hepatoid carcinoma Paneth cell carcinoma Adenocarcinoma with enteroblastic differentiation Parietal cell adenocarcinoma Mucoepidermoid carcinoma Micropapillary adenocarcinoma Adenosquamous carcinoma Squamous cell carcinoma Undifferentiated carcinoma NOS: Large cell carcinoma with rhabdoid phenotype Pleomorphic carcinoma Sarcomatoid carcinoma Carcinoma with osteoclast like giant cells Gastroblastoma <i>Neuroendocrine tumor NOS</i> NET grade 1 NET grade 2 NET grade 3 Gastrinoma NOS Somatostatinoma NOS Enterochromaffin-cell carcinoid ECL-cell malignant carcinoid <i>Neuroendocrine carcinoma NOS</i> Large cell Small cell Mixed neuroendocrine–non neuroendocrine neoplasm (MiNEN)

The World Health Organization (WHO) Classification of Tumors of the Digestive System, published in 2000 based on the views of the Working Group meeting in 1999, classified gastric carcinoma into adenocarcinoma, intestinal and diffuse type, and several subtypes: papillary, tubular and mucinous adenocarcinoma, signet ring cell, adenosquamous, squamous, small cell and undifferentiated carcinoma. This classification had several shortcomings: it was purely descriptive, with little clinical utility; it did not address several subtypes, like mixed diffuse and intestinal type carcinomas, poorly differentiated carcinomas without signet ring cells; and finally, it included small cell carcinomas in the same category with adenocarcinomas, only carcinoid tumors being considered a separate category (Table 1). All these histologic types were recognized by American Joint Committee on Cancer (AJCC) staging manual 5th edition (1997) and staged using the same staging system, except for carcinoid tumors.

Some of the shortcomings were addressed by the subsequent edition of WHO book, published 10 years later. To the previously described categories of papillary, tubular and mucinous adenocarcinoma, a new category was added: poorly cohesive carcinoma, which, included signet ring carcinoma and other variants, as well as mixed adenocarcinoma. Maybe the most important change was separation of neuroendocrine neoplasms as a separate category, which, includes neuroendocrine tumors (NET) grade 1 and grade 2, neuroendocrine carcinoma (NEC), large cell and small cell type, mixed adeno–neuroendocrine carcinoma (MANEC), and NET producing gastrin and serotonin (Table 1). A list of the most common genetic alterations found in intestinal and diffuse types of gastric cancer were included in this edition.

In the meantime, the AJCC staging manual went through two new editions: 6th edition (2002) and 7th edition (2010). Both

editions still included and staged small cell carcinoma together with the other epithelial carcinomas, while carcinoid tumors were a separate category. Finally, the 7th edition created a single separate staging category for well differentiated NET and well differentiated NEC of stomach, small intestine, colorectal and duodenal ampulla.

The latest edition of the WHO book (5th edition), published in 2019, based on the debates of the WHO Classification of Tumors Editorial Board meeting in 2018, significantly expanded the classification of malignant epithelial tumors. Adenocarcinoma was classified in several subtypes: tubular, parietal cell, mixed, papillary NOS, micropapillary NOS, mucoepidermoid, mucinous, signet ring cell, poorly cohesive, medullary carcinoma with lymphoid stroma and hepatoid adenocarcinoma. Undifferentiated carcinoma has several subtypes: large cell carcinoma with rhabdoid phenotype, pleomorphic carcinoma, sarcomatoid carcinoma, carcinoma with osteoclast-like giant cells. Neuroendocrine neoplasms were classified in NET NOS, with the following subtypes: NET grade 1, grade 2, grade 3, gastrinoma, somatostatinoma, enterochromaffin cell carcinoid (ECL) and ECL carcinoid, malignant. Neuroendocrine carcinoma NOS continues to remain a separate category, with large cell and small cell subtypes. MANEC has been renamed as mixed neuroendocrine–non neuroendocrine neoplasm (MiNEN). Gastroblastoma was added as a separate category. Probably the most significant addition was the inclusion of the new genetic and epigenetic molecular classifications proposed by The Cancer Genome Atlas Research Network (TCGA) and the Asian Cancer Research Group (ACRG). The latest edition of the AJCC staging manual (8th edition) published in 2017 and the College of American Pathologists (CAP) Cancer Protocol Templates, which, are frequently updated, created separate pathologic staging systems for well differentiated neuroendocrine tumors, separate for each

gastrointestinal site, while NEC and MiNEN are staged using the same template as carcinomas.

Molecular Classification of Gastric Cancer

Gastric cancer is a highly heterogeneous disease from molecular point of view, in dire need of specific biomarker-driven cancer therapies. There have been several efforts to perform large-scale molecular profiling and classification of GC, summarized in Table 2.

Familial gastric cancer

About 10% of GC show familial clustering, but only approximately 1-3% of GC are associated with inherited gastric cancer predisposition syndromes, such as hereditary diffuse gastric carcinoma (HDGC), familial adenomatous polyposis syndrome (FAP), hereditary nonpolyposis colorectal carcinoma (HNP CC/Lynch syndrome), juvenile polyposis syndrome, Peutz-Jeghers syndrome, Li-Fraumeni syndrome and gastric hyperplastic polyposis syndrome (6).

HDGC is an autosomal dominant disorder with high penetrance. Approximately 30-40% of patients have a germline mutation in the tumor suppressor E-cadherin gene (CDH1). The inactivation of the second allele through mutation, methylation and/or loss of heterozygosity eventually triggers the development of gastric cancer (7-9). Under-expression or complete loss of E-cadherin is associated with epithelial-mesenchymal transition (EMT), a poor prognostic indicator. The presence of this mutation confers over 80% lifetime risk of gastric carcinoma, and prophylactic total gastrectomy after confirmation of CDH1 mutation is the only procedure that increases survival (10). Additionally, annual mammography and breast MRI starting at age of 35 are recommended for women with HDGC, due to their increased risk of lobular breast cancer (11). The histologic phenotype of HDGC corres-

ponds to diffuse type in Lauren classification, or poorly cohesive type with signet ring cells in WHO fifth edition. Relatively characteristic to HDGC in early stage is a pagetoid-like spread of signet ring carcinoma cells (signet ring carcinoma *in situ*), which, has not been reported in the sporadic form of signet ring carcinoma (12). CDH1 genetic testing of blood for germline mutation should be performed in Clinical Laboratory Improvement Amendments (CLIA)-certified molecular diagnostic laboratories in USA, or laboratories with expertise in CDH1 gene analysis.

Beyond hereditary GC, the more common sporadic form of diffuse type GC has also been associated with E-cadherin loss, either through somatic mutation or promoter hypermethylation. Recently, a pair of studies published in *Nature Genetics* expands our understanding of diffuse type GC by describing novel recurrent mutations of *RHOA*, encoding the small GTPase RhoA, in 14.3%–25.3% of patients (13,14). These findings implicate *RHOA* as a novel candidate driver for diffuse gastric cancer.

Human epidermal growth factor receptor 2 (HER2)

HER2 is a member of the epidermal growth factor receptor (EGFR) family, which, includes ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4. All of them are oncogenic drivers and were described initially in lung cancer, breast cancer and glioblastoma, and later in colon cancer, bladder, ovary, cervix, stomach and gastroesophageal junction (GEJ) cancers (15). Inhibitors of these receptors have been associated with the most successful results of targeted cancer therapies to date, including antibody therapeutics (trastuzumab and cetuximab), small molecule tyrosine kinase inhibitors (osimertinib, erlotinib, afatinib, gefitinib, lapatinib, mobocertinib and tucatinib) and the newly developed drug conjugates (trastuzumab emtansine and trastuzumab deruxtecan) (16,17).

Table 2. Current Molecular Classifications of GC

Current molecular classifications of GC					
The Cancer Genome Atlas (TCGA) 2014	EBV EBV-CIMP PIK3CA mutations PD-L1/PD-L2 expression	MSI MLH1 promoter hypermethylation Hypermethylated Gastric CIMP	GS CDH1, RHOA mutations Diffuse histology Younger females	CIN	
				TP53 mutations Intestinal histology	
Singapore-Duke 2013			Mesenchymal	Proliferative	Metabolic
			Low level of CDH1 Low TP53 Diffuse histology PIK3CA-mTOR inhibitors	High TP53 Intestinal histology	Low TP53 No histologic correlation 5-FU+surgery
Asian Cancer Research Group (ACRG) 2015		MSI	MSS/EMT	MSS/TP53-	MSS/TP53+
		ARID1A, KRAS, PIK3CA	Loss CDH1	TP53 mutations, ERB2, EGFR, MDM2, GATA6	EBV, APC, ARID1A, KRAS, PIK3CA, SMAD4
		Intestinal histology Antrum Early stage Best prognosis	Diffuse histology Younger age Worst prognosis	Intestinal histology	Intestinal histology
				Male	Male
				Intermediate prognosis	Intermediate prognosis

HER2 gene amplification and overexpression of the HER2 protein by immunohistochemistry was discovered in gastric carcinoma in 1986 (18). The rate of HER2 overexpression in gastric adenocarcinoma ranges from as low as 4% to as high as 53% (19-23), with an average of 20% in a large meta-analysis (23). HER2 overexpression is more often noted in intestinal type carcinoma adenocarcinomas located at proximal stomach, cardia and gastroesophageal junction. A large phase 3 international clinical trial (ToGA trial) showed that the humanized monoclonal antibody against HER2, trastuzumab (Herceptin), when combined with chemotherapy (capecitabine or 5-FU and cisplatin) prolongs overall survival and

progression free survival in patients with HER2 positive advanced gastric carcinoma (24). Based on this study, in 2010, trastuzumab was approved by FDA in USA, Health Canada and by the European Commission for the treatment of HER2 positive metastatic gastric or GEJ adenocarcinoma in combination with standard chemotherapy, for patients who have not received prior treatment for metastatic disease.

In the ToGA trial, HER2 was assessed by immunohistochemistry and scored as 0, 1+ (negative), 2+ (equivocal) and 3+ (positive). The tumors showing equivocal (2+) staining were confirmed by fluorescent in situ hybridization (FISH), which, if showed amplification of HER2 were considered

positive and patients were eligible for therapy with trastuzumab (Fig.2A). HER2 scoring criteria in GC are slightly different from breast cancer, requiring either complete, basal lateral or lateral membranous staining, unlike breast HER2 interpretation which, requires completely circumferential staining. Another difference between breast and

gastric cancer HER2 interpretations is the amount of tumor cells required: small clusters composed of minimum 5 tumor cells in biopsies of gastric or GEJ cancer, and 10% of tumor cells in resection specimens respectively, are adequate for evaluation (Fig. 2B).

Fig. 2. HER2 testing in gastric cancer: A. Immunohistochemical staining is interpreted as negative (score 0 and 1+), equivocal (score 2+) and positive (score 3+). Tumors with equivocal (2+) staining should be confirmed by fluorescent in situ hybridization (FISH), which, analyzes the ratio between HER2 gene copy number and chromosome 17 centromere. HER2/CEP17 ratio > 2 in over 20 tumor cells is considered HER2 amplified. B. HER2 scoring guidelines in gastric cancer in biopsy and resection specimens, according to the ToGA trial.

Current recommendations are to use immunohistochemistry as the initial testing methodology, with FISH or silver in situ hybridization (SISH) used only to retest equivocal (2+) cases (25). There is a strong demonstrated concordance in HER2 expression between biopsy and resection specimens (26), therefore, most of the time there is no need to re-test HER2 on the resection.

Therefore, HER2 has been established as the first, and, so far, the only approved molecular biomarker available for gastric cancer patients in clinical practice and recommended by CAP (27), and trastuzumab is the first FDA approved molecular targeted agent for the treatment of gastric cancer.

Singapore-Duke Classification of Gastric Cancer

The gastric cancer project 2008 Singapore cohort (28) was one of the first major projects that tried to identify the key oncogenic pathways deranged in gastric cancer, associated with a significant impact on survival. Using gene expression signatures, an *in-silico* strategy to map patterns of oncogenic pathway activation in 301 primary gastric cancers was developed. Also, 3 oncogenic pathways, subsequently validated in gastric cancer cell lines, were described: proliferation/stem cell, NF-kappaB, and Wnt/beta catenin.

A follow-up study of the Singapore cohort conducted by Lei et al. (29) compared gene expression patterns among 248 GC from Singaporean patients and identified 3 major subtypes:

1. proliferative – characterized by high genomic instability, TP53 mutations and DNA hypomethylation
2. metabolic – more sensitive to 5-fluorouracil (5-FU) than other subtypes
3. mesenchymal – with features of cancer stem cells; cell lines of this subtype are particularly sensitive to inhibitors of phosphatidylinositol-3-kinase/mammalian target of rapamycin (PI3K/mTOR) pathway.

The Cancer Genome Atlas (TCGA) Gastric Cancer Molecular Classification

In 2014, as part of TCGA project, the genome and proteome of gastric cancer were extensively characterized in order to uncover molecular subtypes and identify dysregulated pathways and potential therapeutic targets (30). Fresh frozen tissue and matched germline DNA samples of 295 primary gastric adenocarcinoma not previously treated with chemotherapy or radiation therapy were studied by using 6 different molecular platforms (array-based somatic copy number analysis, whole exon sequencing, array-based DNA methylation profiling, mRNA sequencing, micro-RNA sequencing and reverse-phase protein array), with 77% of tumors tested with all 6 platforms.

Analysis of data created a decision tree which, separated the gastric cancer in 4 distinct subtypes, with the following characteristics:

1. Epstein-Barr virus (EBV; 9% of patients)-characterized by EBV positivity, these tumors had higher prevalence of DNA promoter hypermethylation, frequent PIK3CA, ARID1A and BCOR mutations, with rare TP53 mutations. All EBV positive tumors exhibited extreme CpG island methylator phenotype (CIMP), distinct from that in the MSI subtype. They displayed CDKN2A (p16) promoter hypermethylation but lacked the MLH1 hypermethylation characteristic of MSI associated CIMP. Mutations in PIK3CA, ERBB3, ERBB2 and EGFR were noted. EBV gastric cancers were mostly in males and located in the fundus or body and had prolonged survival (31).

2. Microsatellite high instability (MSI; 22% of patients)-included tumors with elevated mutation rates and hypermethylation (including hypermethylation MLH1 promoter). Absent from this group were BRAF mutations, commonly seen in MSI colorectal cancer. These patients tended to be older

females and morphologically, the gastric cancer was intestinal type.

3. Genomically stable (GS; 50% of patients) - the tumor has had a low mutational burden and low somatic copy number aberration. Clinically, these group demonstrated more aggressive disease, with 73% of tumors occurring in younger patients, showing diffuse histology, with CDH1 mutations and RHOA mutations.

4. Chromosomal instability (CIN; 20% of patients) - the tumors show high somatic copy number aberrations, aneuploidy and focal amplification of receptor tyrosine kinases (RTKs) (i.e., EGFR, ERBB2 and ERBB3), and, VEGFA, MET, TP53, APC mutations.

In theory, amplifications of RTKs are amenable to blockade by therapeutics (RTK inhibitors); VEGFA can be targeted by the VEGFR2-targeting antibody (ramucirumab), and amplifications of cell cycle mediators (CCN E1, CCN D1 and CDK 6), are amenable to therapy by inhibition of cyclin-dependent kinases (32). These tumors are more frequent at the gastroesophageal junction/cardia and show intestinal morphology.

Asian Cancer Research Group (ACRG) Gastric Cancer Molecular Classification

The ACRG expanded upon the TCGA analysis by performing additional expression analyses and correlated the results to their institutions high-quality clinical outcomes, which, included recurrence and survival. In its initial study, whole genome sequencing was performed in 49 patients, and recurrent somatic mutations were identified (33). A year later, ACRG published the results of gene expression profiling, genome-wide copy number microarrays and targeted gene sequencing on an additional 251 patients, for a total of 300 primary GC tumor specimens. Tissue was obtained at the time of total or subtotal gastrectomy from Samsung Medical Center in Korea. All patients were

chemotherapy naïve. Long follow-up was available (median 86.4 months). Based on a decision tree, similar with TCGA group, they identified four molecular subtypes that were associated with survival and recurrence patterns after surgery (34):

1. Microsatellite high instability (MSI; 23% of patients) - This subtype had the best prognosis, and more than half of patients were diagnosed with early-stage cancer (stage I–II), similar with TCGA MSI subset. MSI subtype was associated with the presence of hypermutation, with mutations in several genes: K-ras (23.3%), the PI3K-PTEN-mTOR pathway (42%), ALK (16.3%) and ARID1A (44.2%).

2. Microsatellite stable/ epithelial mesenchymal type (MSS/EMT; 15% of patients) - The EMT subtype had a lower number of mutation events compared to the other MSS groups. These tumors were mostly present in young patients (median age 53 years), showed diffuse type histology, presented at advanced stages (stage III–IV) and were more commonly located in the antrum and body. This subtype had the worst overall prognosis and recurrence free survival. Although 80% of MSS/EMT tumors showed diffuse morphology, overall, only 27% of all diffuse gastric cancers in this study were part of this subgroup, which, suggests that more than 70% of diffuse gastric cancer had a better prognosis. This group is similar with the GS group of TCGA. EMT and MSI exhibited a mutually exclusive pattern.

3. MSS/TP53+ (26.3% of patients) - these tumors showed intact TP53, frequent EBV infections and mutations in APC, ARID1A, KRAS, PIK3CA, SMAD4 genes.

4. MSS/TP53- (35.7% of patients) - these tumors showed functional loss of TP53, with the highest prevalence of TP53 mutations (60%) and a low frequency of other mutations: ERBB2, EGFR, MDM2, GATA6. TP53 is the most mutated gene in gastric cancer. TP53 signature was measured in this study based on 2-genes: CDKN1A (p21) and MDM2. Intact TP53 in

group 3 means high signature score (TP53+) whereas functional loss of TP53 means low signature score in group 4 (TP53-). TP53 activity signature correlated with the TP53 mutation status.

As opposed to the TCGA analysis, ACRG group run a survival analysis on their cohort of patients, and they validated on three separate independent cohorts: Samsung Medical Center 2 (n=277); Singapore cohort (n=200); TCGA cohort (n=205). They noticed that the gastric cancer subtypes were statistically correlated to the overall survival in all four cohorts, either individual or combined datasets. The best overall survival was present in the MSI group both in each individual cohort, and in the combined cohorts, while the MSS/EMT subtype had the worst overall survival. The pattern of recurrence for each GC subtype was also investigated as an exploratory analysis using clinical data from the ACRG and Samsung Medical Center cohort. The MSS/EMT group had a higher risk of recurrence compared to the MSI group (63% versus 23%). Additionally, they observed that the first site of recurrence was related to subtypes as follows: MSS/EMT subtype had a higher percentage of peritoneal seeding versus all other subtypes (64% versus 23%); also, the MSI and MSS/TP53 subgroups showed a high percentage of metastases limited to the liver (23% and 21% respectively) versus the MSS/EMT and MSS/TP53+ subtypes (4.6% and 8% respectively). When compared with the other reported molecular subtypes (TCGA and Singapore cohorts), they noticed similarities, such as tumors with MSI in both ACRG and TCGA subtypes, and between the GS, EBV and CIN TCGA subtypes and the ACRG subtypes MSS/EMT, MSS/TP53+ and MSS/TP53 - respectively (Table 2). However, they noticed several differences in terms of cohort, molecular mechanism, driver gene and prognoses association. For example, tumors classified as TCGA CIN subtype were present across all ACRG subtypes. Also, the diffuse type histology was lower in

the TCGA cohort (24% in TCGA versus 45% in ACRG), with most diffuse type cases present in the TCGA GS group, but only 27% cases present in the ACRG MSS/EMT subtype. This is suggestive of less heterogeneity present in the diffuse subtype in the TCGA cohort. Also, CDH1 mutations, frequent in the TCGA GS subtype (37%), were infrequent in the ACRG MSS/EMT subtype (2.8%). Additionally, RHOA mutations were more prevalent in the MSS/TP53- and MSS/TP53+ than in the TCGA GS group. All of these differences suggest that the TCGA GS type is not equivalent to the ACRG MSS/EMT subtype. In general, the Singapore and TCGA subtypes are similar with the notable exception that there is no equivalent subtype that corresponds to the ACRG MSS/TP53+ or MSS/TP53-, making the ACRG classification rather unique. These molecular signatures of gastric cancer have important clinical implications for molecular screening and targeted therapy agents development. However, a significant hurdle for the use of molecular signatures in clinical practice is the cost and availability of these tests.

Several intrepid pathologists have tried to come up with clinically feasible and cost-effective strategies and developed varied subtyping algorithms using immunohistochemical stains and/or FISH, trying to approximate the molecular subtypes of GC. Some of these algorithms are described in the section below.

Development of a practical immuno-histochemical algorithm for the identification of molecular subtypes of gastric cancer

In 2016, Kim et al. had described a ten-stain panel, using EBER, mismatch repair (MMR) proteins (MLH1, PMS2, MSH2, and MSH6), receptor tyrosine kinases (HER2, EGFR, and MET), PTEN, and p53 protein. This panel is extensive, with several immunostains (i.e., EGFR, MET, PTEN) that are not routinely utilized in surgical pathology labs (35).

Fig. 3. Practical pathological algorithms that approximate molecular classification of gastric cancer. A. Integrated classification. B. TCGA classification. EBER in situ hybridization separates EBV group; immunohistochemical stains for MMR or PCR separate MSI group; E-cadherin immunohistochemical stain separate genomically stable (GS) group and the remainder are chromosomal instability (CIN) group. C. ACRG classification. Immunohistochemical stains for MMR or PCR separate MSI group; aberrant E-cadherin stain (complete loss or faint cytoplasmic staining) separate MSS/EMT group; aberrant p53 immunostain (strong staining in >70% of tumor cells) separates the MSS/TP53- group; the remainder group with wild-type (normal) p53 staining pattern is MSS/TP53+ group.

This was a large study conducted on 438 consecutive patients with advanced gastric adenocarcinoma from South Korea. The biomarkers were performed on whole sections slides - a clear advantage over the subsequent studies, the majority performed on tissue microarrays (TMA) blocks, which, include a very small tumor sample (cores of 1 or 2 mm).

The same year, Setia et. al group proposed a 5-tier classification algorithm, based on 14 biomarkers, which, included EBER in situ hybridization and immunohistochemical stains for mismatch repair proteins (MLH1, PMS2, MSH2 and MSH6), E-cadherin, PD-L1, MUC 2, CDX2, CD10,

MUC 5AC, MUC6 and HER2 (36). These biomarkers were performed on TMA blocks constructed from 146 primary gastric cancers. This team described 5 different groups of GC (Fig. 3A):

1. EBER positive (5%) - these tumors showed a prominent lymphoid infiltrate, and they were associated with PD-L1, which, suggests a potential role for immunotherapy with anti-PD1/PD-L1 monoclonal antibodies. EBV cluster showed a trend towards better survival, however not statistically significant.

2. MMR deficient (16%) – which, correlates with the MSI group from both TCGA and ACRG classifications

3. E-cadherin aberrant (21%) - most of the tumors in this group were diffuse type (90%). This group correlates with the genomically stable group of the TCGA, the MSS/ EMT subgroup of the ACRG and the mesenchymal group of the Singapore classification.

4. Aberrant TP53 expression (51%) - further subclassified into intestinal type (33%, based on MUC2 and/or CD10 expression), gastric type (32%, MUC5AC and/or MUC6 positive) and normal type (20%, CD10, MUC2, MUC5AC and MUC6 negative). The subclassification based on MUC and CD10 immunostains has no clinical impact and is purely morphological.

This group was similar to the CIN group of TCGA, the proliferative subtype of Singapore classification and MSS/TP53- of ACRG. The majority (81%) were intestinal type, and a trend of HER2 expression was noted. Gastric cancer with aberrant TP53 expression may benefit from targeted therapy with receptor tyrosine kinase blockers (HER2, EGFR, VEGFR, c-MET), cell cycle (CCNE1, CCND1, CDK6) inhibitors and fibroblast growth factor receptor 2 inhibitors.

5. Normal TP53 expression (wild-type)-corresponding to the metabolic subtype in Singapore classification, is highly sensitive to 5-FU therapy.

These groups are not mutually exclusive; however, they approximate well the recently described genetic classifications of gastric cancer. More importantly, they are easy to perform in most pathology laboratories and they use common immunohistochemical stains and FISH.

Several other authors have attempted to develop a practical algorithm to approximate the molecular classification. In 2019, a Canadian group used 5 commonly used immunohistochemical stains (p53 and MMR proteins MLH1, MSH 2, MSH 6 and PMS 2) and EBV in situ hybridization on TMA blocks constructed from 107 gastric cancers (37). They created a subtyping algorithm based

on TCGA classification, using a series of dichotomizing steps:

1. EBV-CIMP tumors, identified by EBER positivity

2. MSI tumors, based on MMR immunostains

3. CIN

4. GS

The latter 2 groups were based simply on histology (diffuse versus intestinal/mixed types), using the rationale that GS tumors were enriched for diffuse histology in the TCGA classification; HER2 and diffuse histology were mutually exclusive in their cohort. The logic behind this approach was that addition of any other markers would make the subtyping cumbersome and difficult to implement in clinical practice.

The Canadian team also approximated ACRG molecular subtypes based on MMR, which, identifies the MSI subtype; and the microsatellite stable (MSS) tumors were subclassified further using p53, which, identifies the MSS/TP53- and MSS/TP53+ groups. My personal opinion is that this method is elegant and easy to implement in clinical practice; also, it is cost effective and can be performed in any pathology lab.

A large Korean research consortium developed and validated another simplified algorithm as a surrogate for molecular subtypes of gastric cancer, using EBV in situ hybridization, MSI PCR testing and immunohistochemical stains for E-cadherin and p53 on 894 gastric cancers (38). Five successive groups were described: EBV, MSI, E-Cadherin loss (EBV negative/ microsatellite stable/ EMT- like) group, aberrant p53 (EBV negative/ microsatellite stable/ EMT- like/ TP53-) group and normal p53 (EBV negative/ microsatellite stable/ EMT- like/ TP53+) group.

Studies on immunophenotypic classification of GC are currently ongoing. At present, the immunohistochemical definition of GC subtypes, especially related to the TP53 and E-cadherin genes expression, is still unsettled. In general, according to the criteria

of Ando et. al (39), aberrant TP53 expression in tumor cells is demonstrated by strong and diffuse staining of more than 70% of the tumor cells nuclei. A completely negative staining or patchy and weak staining is an indication of a wild type TP53 gene. A complete loss of membranous staining or a faint cytoplasmic reactivity of E-cadherin is indicative of an aberrant expression of this gene. A complete membranous or a heterogeneous cytoplasmic/ membranous staining pattern are considered normal E-cadherin staining patterns (36).

Therapeutic considerations

A better understanding of the various molecular characteristics of gastric cancer is critical for diagnostic assessment, prognostic evaluation, and the development of new therapeutic strategies for this aggressive disease. In GC, heterogeneity is present not only between different patients but, also, significantly, within the same patient where it can be both intra- and inter-temporal and spatial and temporal.

At present, there are several targeted agents FDA-approved for the treatment of gastric cancer: trastuzumab, ramucirumab, pembrolizumab, bemarituzumab, larotrectinib and entrectinib.

Based on the ToGA trial (24), **trastuzumab** was FDA approved in 2010 for patients with HER2 positive tumors for first line treatment in combination with chemotherapy. Based on the REGARD trial (40), **ramucirumab** (a VEGFR2 inhibitor) was FDA approved in 2014 as a second line treatment in patients with unresectable or metastatic disease following treatment with a fluoropyrimidine- or platinum-containing therapy. Ramucirumab is the first biological treatment given that has demonstrated a survival benefit as a single drug in patients with advanced gastric or GEJ adenocarcinoma progressing after first-line chemotherapy. Based on the seminal study by Le et. al (41), **pembrolizumab** (an anti-PD-1) was

approved in 2017 for the treatment of cancer patients with microsatellite instability-high (MSI-H) markers or mismatch repair deficient (dMMR) markers. For gastric cancers expressing PD-L1 and negative for HER2, **pembrolizumab** was approved in 2017 as a third line of treatment in patients with disease progression after two or more systemic therapies, including fluoropyrimidine- and platinum-containing chemotherapy and, if appropriate, HER2/neu-targeted therapy (42, 43). PD-L1 testing as a companion diagnostic test (PD-L1 IHC 22C3 pharmDx) for pembrolizumab, is an immunohistochemical stain performed on formalin-fixed embedded tissue and is scored assessing immunoreactivity of tumor cells and immune cells, using the CPS scoring method (44, 45). The CPS was defined as the total number of tumor cells and immune cells (including lymphocytes and macrophages) stained with PD-L1 divided by the number of all viable tumor cells, then multiplied by 100 (<https://www.captodayonline.com/scoring-gastric-gej-cancers-pd-l1-expression/>). According to the KEYNOTE 059 study, CPS score ≥ 1 predicts response to the PD-1 inhibitors (46, 47).

Additionally, **pembrolizumab** was also approved for solid tumors showing high tumor burden (TMB) who had progressed following prior treatment and lacked alternative treatment options. Based on data from the KEYNOTE-158 study, high TMB was defined as ≥ 10 mutations/Mb by next generation sequencing (NGS), regardless of MSI status (48). TMB is broadly defined as the number of somatic mutations per megabase of analyzed genomic sequences. TMB predicts response to immunotherapy both in MSI-H and MSS populations, and is independent of the level of PD-L1 expression, suggesting a potential role of TMB to identify additional subgroups of patients who may benefit from immunotherapy (49). In GC, the TMB cutoff may vary, from 14 mutations/Mb (50) to 10 mutations/Mb (51) or 8.8 in another study

(52), but it is always associated with better response to immunotherapy.

Based on 3 separate studies (53) in 2018, FDA granted accelerated approval to **larotrectinib** for adult and pediatric patients with solid tumors that have a neurotrophic receptor tyrosine kinase (NTRK) gene fusion without a known acquired resistance mutation, that are either metastatic or where surgical resection is likely to result in severe morbidity, and who have no satisfactory alternative treatments or whose cancer has progressed following treatment. This was the second tissue-agnostic FDA approval for the treatment of cancer after the **pembrolizumab** approval described above. Identification of positive NTRK gene fusion status may be determined in local laboratories using NGS or fluorescence in situ hybridization (FISH). Current ESMO recommendations include pan-TRK immunohistochemistry as an approved method to screen for NTRK gene fusions (54). However, NTRK fusions are extremely rare in GC in Asian patients (55), and one German study found none in 438 Caucasian patients with GC (56), therefore testing for NTRK in GC may not be that useful.

Entrectinib was the third FDA-approved tissue-agnostic drug based on the presence of a biomarker (NTRK) across different types of tumours. Like **larotrectinib**, **entrectinib** is an oral NTRK, ROS1, and ALK inhibitor and appears to be effective against a range of NTRK fusion-positive tumor types. The drug has shown clinically meaningful and durable responses in NTRK-positive tumors. However, similar to **larotrectinib**, it is so rare in GC that its testing is not cost effective.

More recently, based on the FIGHT study (57), in April 2021, FDA granted breakthrough therapy designation to **bemarituzumab** for use in combination with modified FOLFOX6 chemotherapy as first-line treatment in metastatic or locally advanced GC or GEJ adenocarcinoma

fibroblast growth factor receptor 2b (FGFR2b) positive and HER2-negative.

Summary

Pathologists play a crucial role in evaluating the histology and the biomarker results and establishing the appropriate biomarker detection methods. Delineating MSI-H/dMMR tumors and determining HER2 positivity, are straightforward and can be performed in any pathology lab using routine immunohistochemical stains and/or in situ hybridization methods and polymerase chain reaction (PCR) tools. Currently, CAP recommends testing only for HER2 in patients with inoperable, locally advanced, recurrent or metastatic gastric and GEJ adenocarcinomas for whom trastuzumab therapy is being considered, while NCCN guidelines recommend additional universal testing for MSI/MMR by PCR/IHC in all newly diagnosed patients. PD-L1 testing, using an approved FDA companion diagnostic test, may be required for locally advanced, recurrent or metastatic gastric and GEJ adenocarcinomas for whom therapy with PD-1 inhibitors is considered.

The latest advances in molecular methods have increased our understanding of GC biology and led to the development of new comprehensive molecular classifications. The TCGA project was a landmark effort that provided a molecular classification of GC, which, may serve as a valuable adjunct to histopathology. As a result of the TCGA database presence, 4 subtypes with distinct clinical and molecular features of GC were described: EBV, MSI, GS and CIN. However, the TCGA subtypes did not show significant correlations with overall and disease-free survivals (OS and DFS), and this may be due to several factors: the duration of follow-up, the heterogeneity of patients; ethnicities, the geographical distribution and the treatment received. The ACRG group expanded upon the TCGA database analysis by performing additional exper-

sion analyses and correlated the results to their institutions outcomes, which, included both data on survival and recurrence. They also described four subtypes of gastric cancer (MSI, MSS/EMT, MSS/TP53+ and MSS/TP53-), which, unlike the TCGA group classification, were associated with survival and specific recurrence patterns after surgery.

These molecular classifications are time-consuming, extremely complex, costly and require sophisticated molecular technologies, which, prevent their widespread availability and use in clinical practice. Therefore, development of practical, easy to use and cost-effective classification systems for predicting prognosis or treatment response is needed. Immunohistochemical, in situ hybridization and/or PCR ancillary tests are excellent surrogates that approximate the molecular classification.

Currently, several algorithms for phenotyping gastric cancer using immunohistochemical markers and in situ hybridization have been published. Most of the studies done for this purpose employed TMAs, which, sample only a small portion of tumors. A simple and practical algorithm uses EBV in situ hybridization in order to identify EBER, MMR protein expression (using MLH1, MSH2, MSH6 and PMS2), E-

cadherin and p53 immunohistochemical stains (Fig. 3B, C) as surrogate markers for the TCGA and ACRG subgroups. Although they do not match perfectly with the molecular subgroups, all these biomarkers are easily available in any pathology laboratory and represent a cost effective, practical and easy to use algorithm which, approximates genomic profiling and could be an alternative in guiding targeted therapies. They can be used in addition to the already established and approved tests for HER2, PD-L1 and MSI/MMR.

A good communication between the medical oncologists and the pathologists is a must as it facilitates effective tissue collection, proper tissue triage and appropriate histopathological and biomarker testing. Providing a detailed clinical history to the pathologists including information about the clinical stage, the resectability, and the therapy envisioned, may guide the nature of biomarkers that should be tested for. This is especially useful when the amount of tissue is limited, for example, in a mucosal biopsy, where sequential testing for single biomarkers may exhaust the tissue. In these situations, a comprehensive genomic profiling via an NGS validated platform is recommended to identify HER2, MSI/MMR, PD-L1, TMB and NTRK mutations.

Abbreviations:

GC – gastric cancer
 TCGA – the Cancer Genome Atlas project
 ACRG – Asian Cancer Research Group
 PCR – polymerase chain reaction
 US – United States
 HDGC – hereditary diffuse gastric cancer
 WHO – World Health Organization
 AJCC – American Joint Committee on Cancer
 NET – neuroendocrine tumors
 NEC – neuroendocrine carcinoma
 MANEC – mixed adeno–neuroendocrine carcinoma
 NOS – not otherwise specified
 ECL – enterochromaffin cell carcinoid
 MiNEN – mixed neuroendocrine–non neuroendocrine neoplasm
 CAP – College of American Pathologists
 FAP – familial adenomatous polyposis syndrome

HNP CC – hereditary nonpolyposis colorectal carcinoma
 EMT – epithelial–mesenchymal transition
 CLIA – Clinical Laboratory Improvement Amendments
 GEJ – gastroesophageal junction
 FDA – The United States Food and Drug Administration
 SISH – silver in situ hybridization
 5-FU – 5-fluorouracil
 PI3K/mTOR – phosphatidylinositol-3-kinase/mammalian target of rapamycin
 MSI – microsatellite high instability
 GS – genomically stable
 CIN – chromosomal instability
 RTK – receptor tyrosine kinases
 EBV – Epstein-Barr virus
 MSS – Microsatellite stable
 EMT – Epithelial mesenchymal transition
 MMR – mismatch repair
 EBER – Epstein-Barr virus-encoded small RNAs
 FISH – fluorescence in situ hybridization
 TMA – tissue microarray
 MSI-H microsatellite instability-high
 dMMR – mismatch repair deficient
 CPS scoring – combined positive score
 PD-1/PDL-1 – programmed cell death protein 1/ programmed cell death-ligand 1
 TMB – high tumor burden
 NTRK – neurotrophic receptor tyrosine kinase
 VEGFA – vascular endothelial growth factor A
 VEGFR – vascular endothelial growth factor receptor
 NGS – next generation sequencing
 ESMO – European Society of Medical Oncology
 FGFR2b – fibroblast growth factor receptor 2b
 OS – overall survival
 DFS – disease free survival

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