

REVIEW

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Is there still a place for conventional histopathology in the age of molecular medicine? Laurén classification, inflammatory infiltration and other current topics in gastric cancer diagnosis and prognosis

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Summary. Gastric cancer (GC) is the fifth most common cancer and the third cause of cancer-related deaths worldwide. In western countries, more than half of GC patients are diagnosed at advanced stages and 5-year survival rates range between 20-30%. The only curative treatment is surgery, and despite recent advances in oncological therapies, GC prognosis is still poor. The main prognostic tool for patient categorization and treatment selection is the TNM classification, but its limitations are being increasingly recognized. Early recurrences may occur in early-stage disease, and patients at the same stage show heterogeneous outcomes. Thus, there is a need to improve GC stratification and to identify new prognostic factors, which may allow us to select drug-susceptible populations, refine patient grouping for clinical trials and discover new therapeutic targets. Molecular classifications have been developed, but they have not been translated to the clinical practice. On the other hand, histological assessment is cheap and widely available, and it is still a mainstay in the era of molecular medicine. Furthermore, histological features are acquiring new roles as reflectors of the genotype-phenotype correlation, and their potential impact on patient management is currently being analyzed. The aim of this literature review is to provide a modern overview of the histological assessment of GC. In this study, we discuss recent topics on the histological diagnosis of GC, focusing on the current role of Laurén classification and the potential value of new histological

features in GC, such as inflammatory infiltration and tumor budding.

Key words: Gastric, Laurén, Budding, Inflammation, Prognosis

Introduction

Gastric cancer (GC) is the fifth most common cancer and the third cause of cancer-related deaths worldwide (Bray et al., 2018). In 2018, more than a million cases were diagnosed and 783000 deaths due to GC were registered. It shows significant geographic variation, being more prevalent in Asia and East Europe. GC incidence and mortality seem to be declining in the last years (Ang and Fock, 2014), which could be due to a decrease in *Helicobacter pylori* infection and improved food preservation (Tang et al., 2020). However, GC in young patients, proximal GC and diffuse tumors are becoming more frequent (Arnold et al., 2020; Petryszyn

Abbreviations. ACRG, Asian Cancer Research Group; CIN, chromosomal instability; CLR, Crohn's like lymphoid reaction; CRC, colorectal cancer; CTLA4, cytotoxic T-lymphocyte associated protein 4; DFS, disease-free survival; EBV, Epstein-Barr virus; EMT, epithelial-to-mesenchymal transition; EUS, endoscopic ultrasound; GC, gastric cancer; GS, genomically stable; HIPEC, hyperthermic intraperitoneal chemotherapy; IHC, immunohistochemistry; IT, immunotherapy; ITBCC, International Tumor Budding Consensus Conference; LP, linitis plastica; MRI, magnetic resonance imaging; MSI, microsatellite instability; NK, natural killer; OS, overall survival; SRCG, signet-ring cell gastric cancer; TAMs, tumor-associated macrophages; TANs, tumor-associated neutrophils; TB, tumor budding; TCGA, The Cancer Genome Atlas; TILs, tumor-infiltrating lymphocytes; UICC, Union for International Cancer Control; WHO, World Health Organization

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et al., 2020).

GC can be divided into proximal and distal types, and these tumors show different risk factors and incidence over time. *H. pylori* infection has been considered the main risk factor for GC development, especially for distal GC (Petryszyn et al., 2020). As previously stated, *H. pylori* infection is diminishing, and other factors such as autoimmune gastritis, changes in gastric microbiota or prolonged treatment with proton pump inhibitors are gaining importance (Anderson et al., 2018).

Despite recent advances in therapeutic regimens, GC prognosis remains very poor. Screening programs have been developed in high-incidence countries, where GC is diagnosed at earlier stages and shows better prognosis (Hamashima et al., 2018). However, in western countries more than a half of GCs are diagnosed at advanced stages, with 5-year survival rates of 20-30% (Charalampakis et al., 2018).

The main prognostic tool for patient stratification and treatment selection is the TNM classification, which includes T stage (tumor depth), N (lymph node metastasis) and M (distant metastasis). The limitations of the TNM system are being discussed in the current literature. Prognostic outcomes of patients at the same stage are heterogeneous, and several authors have reported early recurrences in cases of initial GC (Eo et al., 2015; Rawla and Barsouk, 2019). Lymph node status is recognized as one of the best indicators of poor prognosis in patients with resected GC. The last TNM classification is based on the number of metastatic lymph nodes, but it does not assess LN regions or the total number of LN resected (Wittekind, 2015). Therefore, several alternative lymph node staging systems have been recently published (Choi et al., 2016).

Other prognosticators have been described, but they have not been implemented in practice. Among these factors are clinical (age, gender, nutritional status, performance status, blood test variables), histological, immunohistochemical (IHC) and molecular features (Costa et al., 2006). The Cancer Genome Atlas and the Asian Cancer Research Group have published different molecular classifications for GC, with a huge impact on the understanding of GC pathogenesis and heterogeneity, but these classifications have not been translated yet to the clinical practice (Cancer Genome Atlas Research Network, 2014; Cristescu et al., 2015). Molecular techniques are expensive and they are not widely available, and IHC surrogates for molecular classifications have not been standardized. In contrast, histological assessment is a cheap and widespread method. Several histological features apart from the TNM system have demonstrated significant prognostic value in previous studies, and they may be helpful to stratify GC patients (Fuchs et al., 2017). Moreover, the role of new histological features in GC, such as tumor budding or inflammatory response, is being investigated (Zheng et al., 2017; Guo et al., 2019).

The only curative treatment for GC is surgery, and

resectable tumors are treated by total or subtotal gastrectomy (Smyth et al., 2016). Small and non-ulcerated pT1a tumors with low grade histology can be treated by endoscopic submucosal dissection or endoscopic mucosal resection. Although D2 lymphadenectomy is recommended as a standardized procedure, the extent of lymphadenectomy is a matter of debate. In fact, the number of D2 lymphadenectomies performed in western countries is still low (Zhou et al., 2016).

In the era of personalized medicine, the identification of potential targets in GC is urgently needed. Trastuzumab (anti-HER2) and ramucirumab (anti-VEGFR2) are the only non-chemotherapeutic drugs approved specifically for GC treatment (Digkila and Wagner, 2016). Thus, the assessment of HER2 status by IHC and/or in situ hybridization techniques is mandatory in cases of advanced GC. Apart from these drugs, the agnostic approval of Pembrolizumab (anti programmed cell death protein 1 -PD1-) for microsatellite-high solid tumors also applies to GC (Boyiadzis et al., 2018). Nivolumab (anti-PD1) combinations are being studied, and it has been approved in Asian countries for advanced GC treatment in third-line therapy (Hirotsu et al., 2019). The improvement in GC stratification and the identification of new prognostic factors may lead to better results in clinical trials, because an optimal grouping strategy is essential for identifying drug-susceptible populations and increasing patient subclassification, homogenization and comparability.

The aim of this literature review is to provide a modern overview of the histological assessment of GC in the age of molecular medicine. In this study, we discuss recent topics on the histological diagnosis of GC, focusing on the current role of Laurén classification and the potential value of new histological features in GC, such as inflammatory infiltration and tumor budding.

Laurén classification

Definition and epidemiology

The Laurén classification was published in 1965, and it has been widely adopted by pathologists and clinicians (Laurén, 1965). According to this classification, GC can be divided into intestinal, diffuse and mixed types. Intestinal tumors show glandular lumina, often accompanied by papillary fronds or solid structures (Fig. 1). Diffuse GC is composed of scattered carcinoma cells, as solitary cells or small clusters, growing in a diffuse and highly infiltrative pattern (Fig. 2). The authors found a correlation between histotypes and clinical features of GC patients. Intestinal-type tumors are more frequent in men and in older patients, they occur as polypoid or fungating masses, and they are associated with the sequence of chronic atrophic gastritis - intestinal metaplasia - dysplasia. Intestinal GC is related to *H. pylori* infection and environmental factors

(Marrelli et al., 2018). Diffuse tumors usually occur in younger patients and they may be induced by active inflammation and/or genetic factors (Wang et al., 2016a; Tang et al., 2020).

As for the epidemiological features, intestinal GC is more common than diffuse GC. Diffuse GC is equally distributed between geographic locations, and intestinal GC is more prevalent in high-risk countries (Min et al.,

2017). Chen et al. found an intestinal to diffuse type ratio of 1.42 in Asian population (Chen et al., 2016). Current epidemiological studies indicate that the worldwide incidence of GC is declining, and intestinal-type tumors show a more pronounced decrease than diffuse tumors. So, the incidence of diffuse GC and GC in young patients is relatively increasing in the last years (Tang et al., 2020).

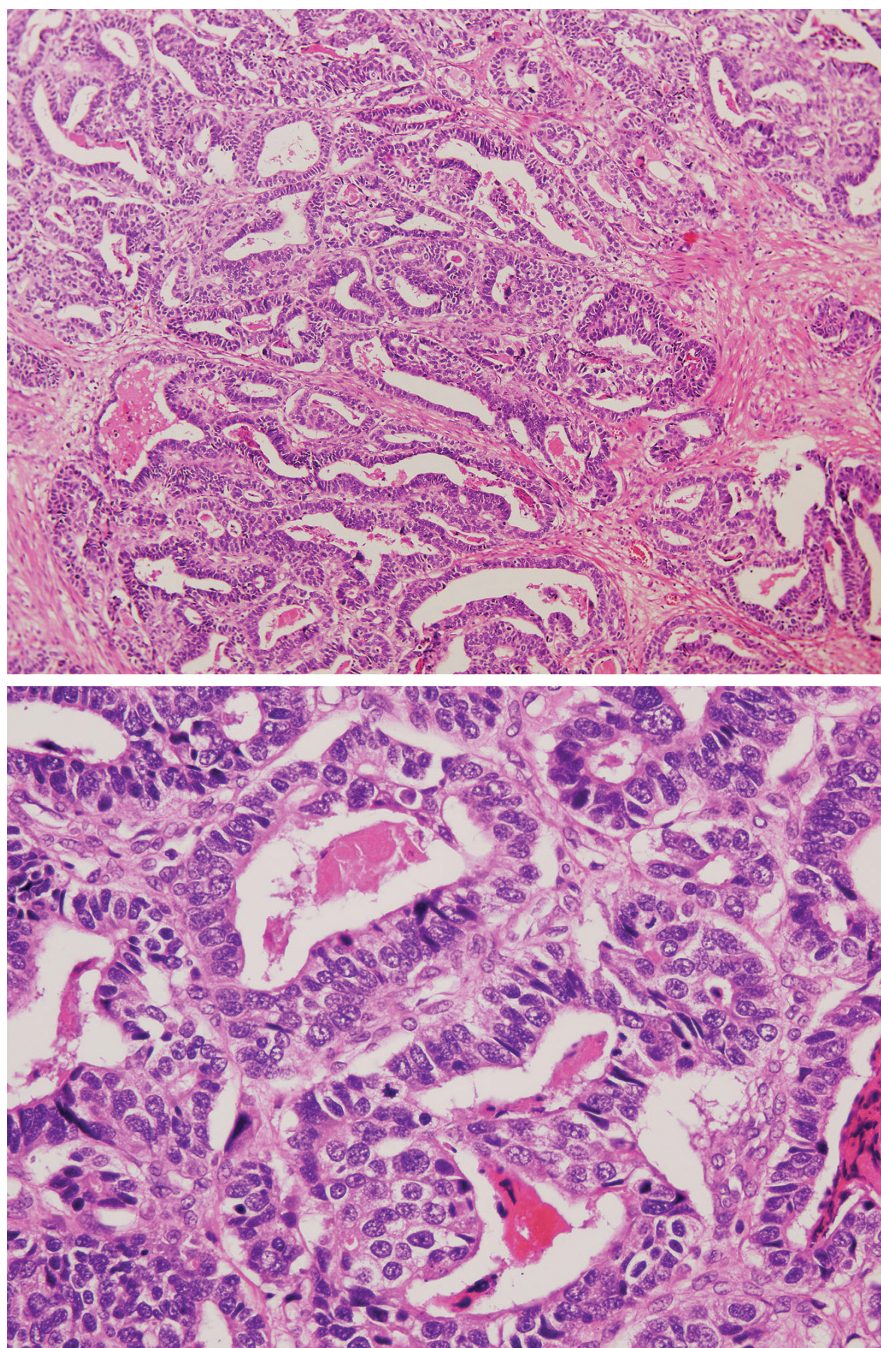


Fig. 1. Intestinal gastric cancer. Glandular structures with intraluminal secretion. Hematoxylin-eosin staining.

Prognostic value

Laurén classification is still considered an important prognostic factor in GC patients, as seen in recent studies (Chen et al., 2016; Wang et al., 2016a, 2018; Luu et al., 2017; Petrelli et al., 2017; Lee et al., 2018a; Tang et al., 2020). Most authors identified Laurén classification as an independent prognosticator of GC, and observed that diffuse tumors are associated with poorer prognosis (Chen et al., 2016; Wang et al., 2018). Diffuse-type GC shows higher rates of lymph node and distant metastases, higher T stage and higher perineural infiltration (Fig. 3) (Luu et al., 2017; Zhao et al., 2018). However, other investigators reported a similar or better prognosis for diffuse tumors (Pyo et al., 2016; Lee et al., 2017a) or did not recognize Laurén classification as an independent prognosticator (Roy et al., 1998). A recent meta-analysis including 73 publications with more than

61000 patients indicated that Asiatic and Western patients with diffuse-type morphology have worse prognosis in both loco-regional and advanced disease (Petrelli et al., 2017). Laurén subtype was an independent prognostic factor in the multivariate analysis, and the prognostic effect of the Laurén classification was not influenced by the administration of neoadjuvant therapy.

In a study including 957 patients with resected GC, Laurén subtype was the most important factor associated with the pattern of recurrence (Lee et al., 2018a). Diffuse tumors showed peritoneal (37%), distant (32%) and locoregional (22%) recurrences. Patients with intestinal GC had distant (54%), locoregional (20%) and peritoneal (15%) recurrences. Diffuse tumors may have a great ability to migrate, develop epithelial-to-mesenchymal transition, and their cells can interact with the microenvironment on the peritoneal surface

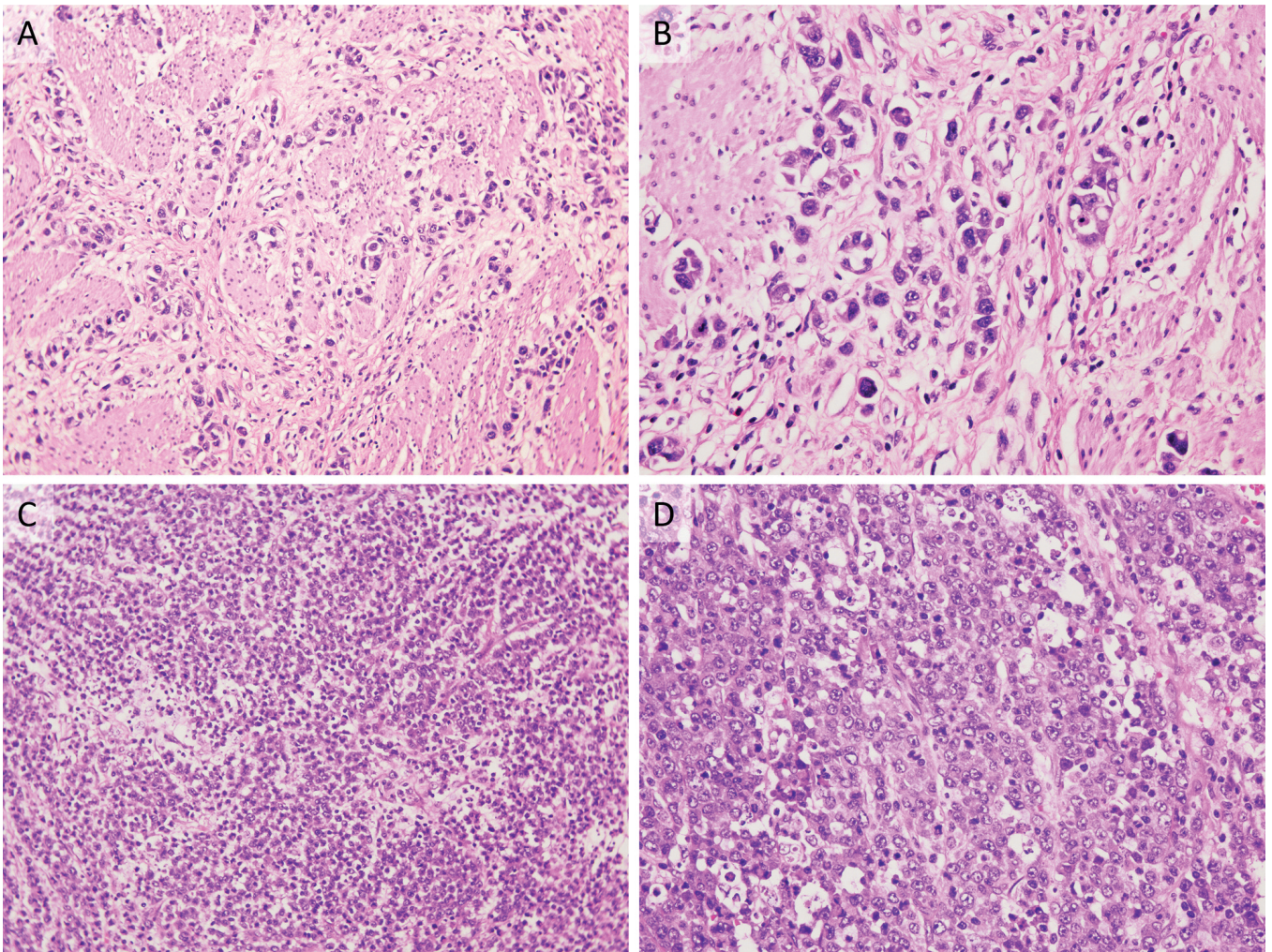


Fig. 2. Diffuse gastric cancer. **A, B.** Tumor cells infiltrating the gastric wall as isolated cells, small cords, strands and clusters. **C, D.** Sheets of monotonous tumor cells with no glandular formation. Hematoxylin-eosin staining.

(Toiyama et al., 2012; Kinoshita et al., 2015).

Finally, the role of a new tumor location-modified Laurén classification system has been evaluated (Shah et al., 2011; Choi et al., 2015; Zhao et al., 2018). According to this classification, GC is divided into proximal non-diffuse, diffuse and distal non-diffuse. Non-diffuse tumors include both intestinal and mixed GC, and proximal tumors are those whose bulk is located in the gastric cardia. Proximal tumors are more aggressive and show poor prognosis. This combined system is an independent prognostic factor for GC, and it has demonstrated better survival predictive accuracy and discriminatory ability than the Laurén classification.

Molecular features

The technological advances experienced in the last years have allowed us to investigate the biological

heterogeneity of solid tumors and to develop molecular classifications (Cisło et al., 2018). This knowledge could be helpful to refine patient stratification, to identify new molecular targets and to improve treatment algorithms. The most important molecular classifications in GC have been published by The Cancer Genome Atlas (TCGA, 2014) and the Asian Cancer Research Group (ACRG, 2015) (Table 1). The classification of TCGA divides GC into four groups: Epstein-Barr virus (EBV, 9%), microsatellite instability (MSI, 22%), chromosomal instability (CIN, 50%) and genomically stable (GS, 20%) (Cancer Genome Atlas Research Network, 2014). The ACRG classification defined four subtypes: MSI (23%), mesenchymal (15%), p53 inactive (36%) and p53 active (27%) (Cristescu et al., 2015). Molecular subtypes show different clinicopathological and prognostic features (Marrelli et al., 2018). Interestingly, an association between Laurén subtypes and molecular

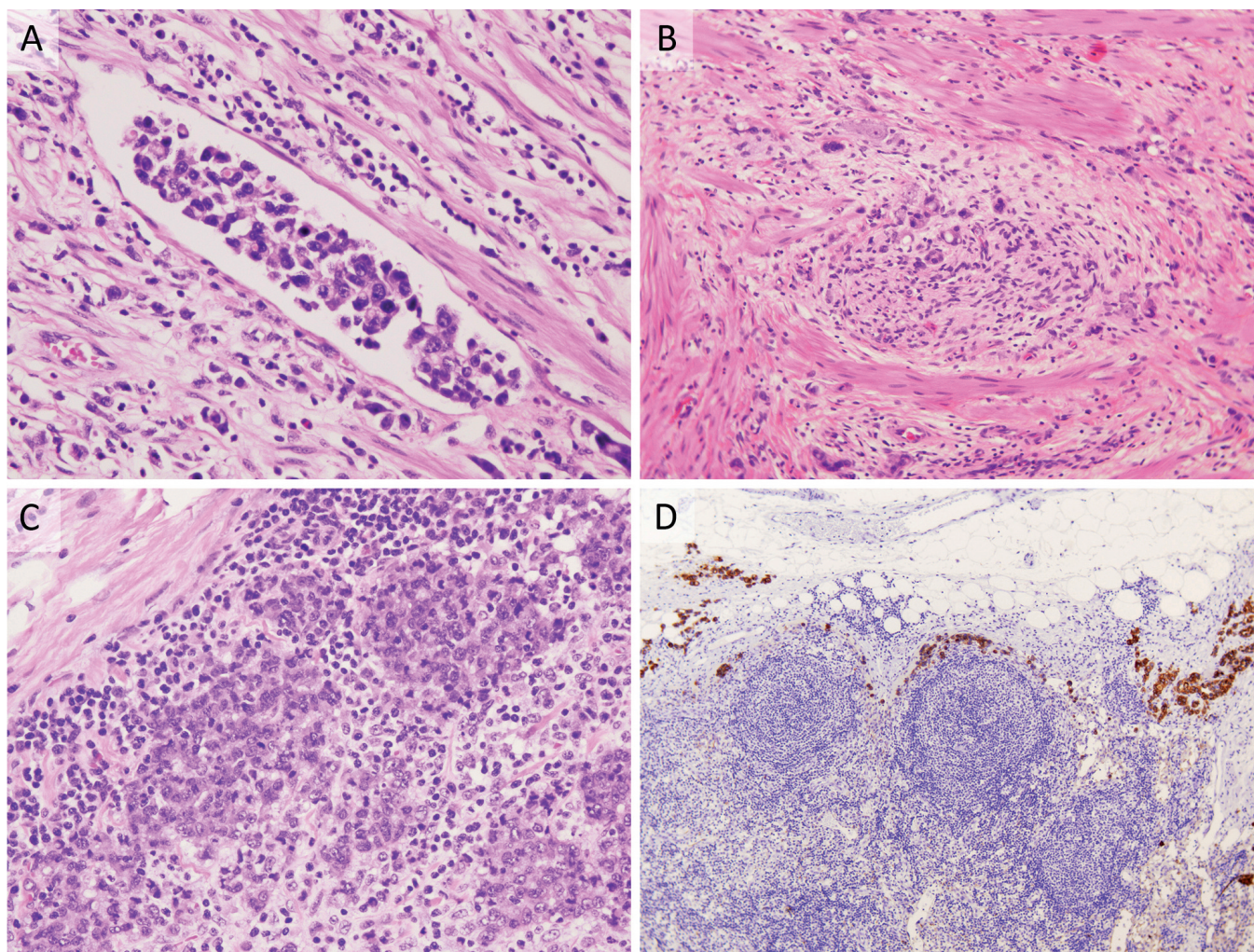


Fig. 3. Diffuse gastric cancer, histological features. **A.** Vascular invasion. **B.** Perineural infiltration. **C, D.** Lymph node metastasis. Hematoxylin-eosin (**A-C**) and CKA61-AE3 (**D**) staining.

classifications has been observed. Most MSI and CIN tumors are intestinal-type GC. In contrast, mesenchymal and GS tumors are mainly diffuse. So, intestinal and diffuse tumors exhibit different genomic profiles. Intestinal tumors are related to MSI, promoter region methylation, higher mutation rates (TP53, ARID1A, PIK3CA, FAT4, HER2, KRAS, APC), and higher copy-number alterations (tyrosine kinase receptors, cell cycle genes) (Oue et al., 2019). A recent metaanalysis further confirmed the relationship between intestinal morphology and MSI (Polom et al., 2018). Cell adhesion molecules or apoptotic genes are overexpressed in this subtype (Zhang et al., 2016). Diffuse GC is associated with CDH1 mutation and shows fewer mutations (TP53, ARID1A, FAT4, RHOA) and less copy-number alterations (FGFR, MET) (Oue et al., 2019). Approximately 9% of diffuse GC cases in western countries are MSI (Hirotsu et al., 2019). Cell adhesion molecules and extracellular matrix components are upregulated in diffuse GC (Zhang et al., 2016).

Moreover, specific gene signatures for Laurén subtypes have been developed. A 40-gene signature was created based on microarray data of 300 patients (Min et al., 2017). In another study, the authors identified a 9-gene set including genes differentially expressed and associated with favorable prognosis in intestinal GC and adverse prognosis in diffuse GC (Zhang et al., 2016). Thus, patients with low intestinal or high diffuse signature scores had lower overall survival (OS) and disease-free survival (DFS) rates. A three-gene signature for prognostic prediction in diffuse GC has also been

developed, including C-C motif chemokine 11 (CL11), collagen type IV alpha 5 chain (COL4A5) and endomucin (EMCN) genes (Bao et al., 2019). The investigators found that EMCN and COL4A5 may be involved in the process of peritoneal metastasis through Wnt and integrin-focal adhesion kinase (FAK) signaling pathways. Finally, a 7 gene signature can predict OS in intestinal-type GC (Binato et al., 2018).

As previously stated, HER2 status analysis is mandatory in advanced GC. Laurén classification also plays a role in this context, because intestinal tumors have higher HER-2 positivity than diffuse GC (Baretton et al., 2019). The association between vascular endothelial growth factor receptor 2 (VEGFR-2) or VEGF expression and Laurén subtypes is controversial, and some studies did not find significant differences between Laurén classification and VEGF or VEGFR-2 expression (Li et al., 2019). However, other investigators have reported that intestinal GC may be more dependent on angiogenesis than diffuse GC (Kitadai, 2010).

Treatment

As for surgical management, diffuse tumors usually need wider surgical margins to achieve an R0 resection, and a super-extended lymphadenectomy might be the best surgical approach (Marrelli et al., 2018). A survival benefit with D3 lymphadenectomy, compared with D2 lymphadenectomy, can be obtained in diffuse and mixed GC (De Manzoni et al., 2015). The indication for endoscopic treatment generally depends on the Laurén

Table 1. Molecular classifications of gastric cancer developed by The Cancer Genome Atlas (TCGA) and the Asian Cancer Research Group (ACRG). Correlation between molecular subtypes.

TCGA	ACRG
MICROSATELLITE INSTABILITY (22%)	MICROSATELLITE INSTABILITY (23%)
Older, female patients	Intestinal type in 60% of cases
Distal location	Distal location
High mutation rate: PIK3CA, ERBB3, ERBB2, EGFR	Better prognosis
Promoter hypermethylation	DNA hypermethylation
No V600E BRAF mutation	High mutation rate: KRAs, PIK3CA, ALK
GENOMICALLY STABLE (20%)	MESENCHYMAL (15%)
Diffuse-type	Diffuse type in 80% of cases
Younger patients	Younger patients
Distal location	Poor prognosis
RHOA and ARID1A mutations	Loss of CDH1 expression
CLDN18-ARHGAP26 fusion	Fewer mutations and amplifications
CHROMOSOMAL INSTABILITY (50%)	P53 INACTIVE (36%)
Intestinal-type	Intermediate prognosis
Proximal location	Copy number alterations, such as ERBB2, EGFR, KRAS or MET amplifications
Amplification of RTK ^a and cell cycle-related genes	TP53 mutation in 60% of cases
Mutations in TP53, ARID1A, KRAS, PIK3CA	
EPSTEIN-BARR VIRUS (9%)	P53 ACTIVE (27%)
Young, male patients	Intermediate prognosis
PIK3CA mutation	Mutations in APC, PIK3CA, KRAS, ARID1A
ERBB2 and JAK2 amplifications	TP53 mutation in 23.7% of cases
PDL1 and PDL2 overexpression	
DNA hypermethylation	

subtype: the usual criteria include intestinal histology, size <20 mm, tumor confined to the mucosa and absence of lymphatic or venous invasion (Lee et al., 2016; Ronellenfitsch et al., 2016). Due to the higher frequency of peritoneal involvement, a staging laparoscopy should be considered in these tumors (Rawicz-Pruszyński et al., 2019). In addition, diffuse GC may benefit from prevention and/or treatment of peritoneal metastases using hyperthermic intraperitoneal chemotherapy (HIPEC) (Ma et al., 2016b; Marrelli et al., 2018).

In respect of chemoradiotherapeutic regimens, most clinical trials and treatment algorithms are based on the TNM classification, and do not stratify patients according to their histotype. Some authors have highlighted the need for including other features beyond disease stage, and Laurén classification could be a simple and cost-effective surrogate marker of GC biology and heterogeneity (Petrelli et al., 2017). After the discovery of the molecular landscape and the histological-molecular correlation of GC, several studies have specifically evaluated the role of Laurén classification for treatment selection.

Response to adjuvant therapy may vary depending on Laurén subtypes. We have summarized the main data of clinical trials and comparative studies analyzing chemotherapeutic regimens and Laurén subtypes in Table 2 (Yamaguchi et al., 2002; Ajani et al., 2010; Narahara et al., 2011; Kato et al., 2012; Lordick et al., 2013; Jiménez Fonseca et al., 2017; Cheng et al., 2019). Most retrospective studies, comparative studies and subgroup analyses of clinical trials found that intestinal

GC is more chemo-sensitive than diffuse GC (Zurlo et al., 2020). Fonseca et al. observed that Laurén subtypes respond differently to chemotherapy in western patients: in their series, anthracycline or docetaxel schedules increased overall response rate only in intestinal tumors (Jiménez Fonseca et al., 2017). Furthermore, the last drug increased both OS and DFS only in intestinal GC. On the other hand, several studies on Asiatic populations suggest that S-1, paclitaxel and irinotecan may be more active in patients with diffuse GC, as seen in Table 2. Only one meta-analysis, published in 2020, has analyzed the impact of the Laurén classification on the response to adjuvant treatment (Wang et al., 2020a). It included data from 33 studies and more than 10000 patients. The authors found that Laurén subtype should be considered when prescribing chemotherapy in GC patients, because diffuse GC is more resistant to standard chemotherapeutic regimens than intestinal GC.

As for perioperative therapy, Zurlo et al. hypothesized that the benefit of perioperative chemotherapy may be limited to the intestinal subtype in locally advanced GC, because patients with intestinal morphology survive longer than patients with diffuse GC (Zurlo et al., 2020). Laurén subtype has been identified as an independent prognosticator of OS in patients treated with neoadjuvant therapy (Hu et al., 2019). Other authors have reported that major histopathological regression is less frequent in non-intestinal tumors (Becker et al., 2011) and these patients also show poorer survival rates after neoadjuvant treatment (Persiani et al., 2005; Wang et al., 2016b).

Table 2. Adjuvant chemotherapy regimens and Laurén subtypes: clinical trials and comparative studies.

Author, year	Pop ^a	Findings
Ajani et al., 2010	Asia	Cisplatin/S1 vs cisplatin/infusional fluorouracil (phase III) No significant differences in survival (whole cohort) Subanalysis: diffuse tumors showed longer OS
Narahara et al., 2011	Asia	Irinotecan/S1 vs S1 (phase III) Irinotecan/S1 patients achieve longer OS (not significant) Subanalysis: diffuse tumors showed longer OS (significant)
Yamaguchi et al., 2002	Asia	Paclitaxel (3h infusion) (phase II) Effective and well tolerated (whole cohort) Diffuse tumors showed higher response rates (36% vs 24%)
Kato et al., 2012	Asia	NK105 (paclitaxel-incorporating micellar nanoparticle) (phase II) Modest effectiveness and tolerability (whole cohort) Diffuse tumors showed higher overall response rate (32% vs 20%)
Fonseca, 2017	West	AGAMENON national registry Anthracycline and docetaxel combinations increased overall response rate only in intestinal tumors Docetaxel increased overall survival and disease-free survival only in intestinal tumors
Lordick et al., 2013	A+W	Capecitabine/cisplatin/cetuximab vs capecitabine/cisplatin (phase III) Diffuse (significant) and mixed (not significant) tumors showed better DFS with capecitabine/cisplatin Diffuse and mixed tumors showed better OS with capecitabine/cisplatin (not significant)
Cheng et al., 2019	Asia	Oxaliplatin-based vs oxaliplatin-free (retrospective) Intestinal tumors showed significantly better OS and DFS with oxaliplatin Diffuse tumors did not show differences between groups

^a: Population: W (western), A (asian).

However, no randomized trial has specifically evaluated the role of perioperative therapy depending on Laurén subtypes.

A. Mixed type

Fewer studies have analyzed the behavior and clinical features of mixed tumors. Mixed GC exhibits both intestinal and diffuse morphology in the resection specimen. It may be difficult to reach a diagnosis of mixed GC in biopsy specimens, because small samples can be not representative of the whole tumor. Approximately 20-30% of GC cases are mixed tumors, and clinicopathological features of mixed GC are similar to that of diffuse GC (Yoon et al., 2016). Mixed tumors present larger size, more lymph node metastases and poorer prognosis than intestinal-type GC (Chen et al., 2016). Some authors have even reported a higher rate of

lymph node metastases for mixed tumors than for diffuse GC (Kozuki et al., 2002). On the basis of these results, mixed tumors could be included in the diffuse type of the Laurén classification. However, the translation of these findings to the clinical setting, especially for patient selection for endoscopic treatment, is controversial, and the definition of “mixed tumor” varies widely in the published literature (Park et al., 2016; Yoon et al., 2016). In respect of its molecular biology, mixed GC shows increased expression of Ki-67, extracellular matrix metalloproteinase inducer (EMMPRIN) and VEGF, and it is associated with CpG island hypermethylation (Saito et al., 2001; Park et al., 2010).

B. Signet-ring cell carcinoma

Signet-ring cell GC (SRCG) is included in the

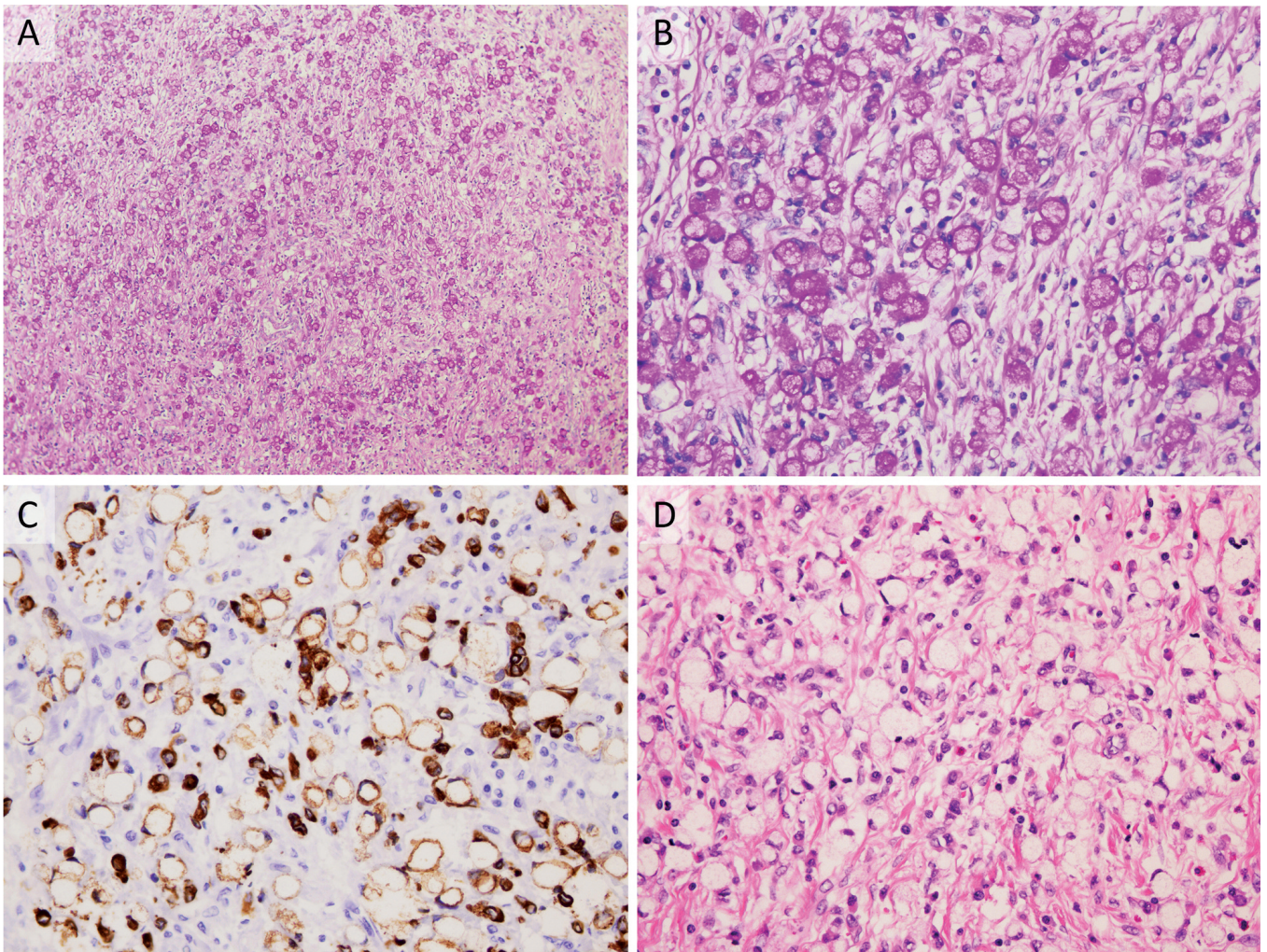


Fig. 4. Signet ring cell carcinoma. Cells with abundant intracytoplasmic mucin and small peripheral nuclei diffusely infiltrating the gastric wall. Periodic acid-Schiff (A, B), CKAE1-AE3 (C) and hematoxylin-eosin (D) staining.

diffuse-type of the Laurén classification. However, recent studies have assessed the particular features of these tumors, and there is controversy whether to consider SRCG as a distinct entity or a subtype of diffuse or poorly cohesive GC (Voron et al., 2016). In SRCG, intracellular mucin pushes the nucleus of the cell to the periphery in more than 50% of cells (Fig. 4) (Ma

et al., 2016b). The rate of SRCG seems to be increasing in the last years (Henson et al., 2004; Anderson et al., 2010). It is more frequent in young, female patients and it usually occurs in the middle and lower third of the stomach (Chon et al., 2017; Nie et al., 2017).

SRCG has been associated with a worse prognosis, higher stage at diagnosis, higher rates of peritoneal

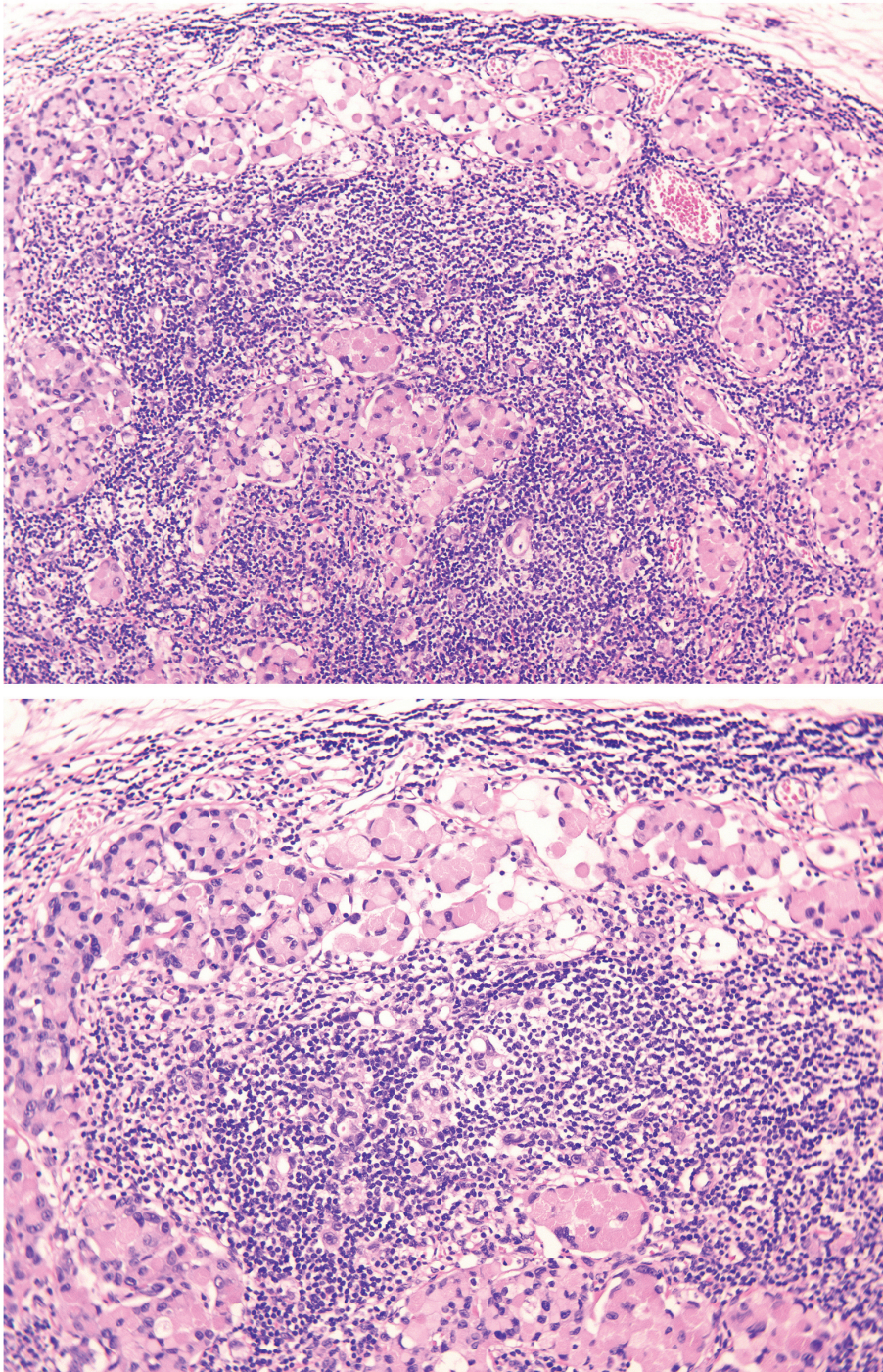


Fig. 5. Lymph node metastasis, signet-ring cell carcinoma. Hematoxylin-eosin staining.

carcinomatosis, lymph node metastases and a lower rate of R0 resections in GC patients (Fig. 5) (Piessen et al., 2009). However, some investigators did not find survival differences between SRC and non-SRC patients (Taghavi et al., 2012). Other authors have not identified SRCG as an independent prognosticator (Postlewait et al., 2015). Current studies suggest that the prognostic impact of SRC may depend on the TNM stage, because early SRCG shows better prognosis than other GC subtypes (Kao et al., 2019), and advanced SRCG shows the worst prognosis; this was further confirmed by a recent meta-analysis (Nie et al., 2017). Early SRCG may represent a latent and low aggressive state, and tumor invasiveness may be accelerated when cancer cells infiltrate the muscular wall, leading to lymph node metastases and peritoneal involvement (Gronnier et al., 2013).

Several features have been recognized as prognosticators in SRCG patients: age, sex, tumor size, depth or lymphovascular invasion (Zhao et al., 2017; Zhou et al., 2019). Young patients present with a more advanced disease and show more distant metastases (Zhou et al., 2020), but some authors have observed a relationship between younger age and improved survival (Ren et al., 2018). A recent study found that the intratumoral stroma can predict SRCG prognosis, and tumors with high stromal proportion are associated with lower DFS and OS (Lee et al., 2017b). Finally, perineural infiltration may contribute significantly to peritoneal recurrence in SRCG (Lee et al., 2018b).

As for SRCG treatment, a recent review summarizes the evidence regarding SRC management (Mengardo et al., 2018). The recommendations are almost the same as for diffuse tumors and linitis plastica. Endoscopic resection should be carefully contemplated due to the higher rate of lymph node metastases, although some investigators have reported that it is safe to perform endoscopic treatment in SRCG and it may be indicated in early tumors (Kang et al., 2017; Kim et al., 2020). The recommended surgical approach includes wider margins than for intestinal type cancer, a D3 lymphadenectomy could be considered, and the role of HIPEC should be evaluated. SRC tumors do not respond well to chemotherapy (Cunningham et al., 2006; Ychou et al., 2011). The use of radiotherapy was less effective in diffuse tumors in previous clinical trials (Park et al., 2015), but a recent study showed that postoperative radiotherapy improves survival in SRCG (Wei et al., 2019). Moreover, a subgroup of patients could benefit from palliative gastrectomy and chemotherapy (Shi et al., 2019). Neoadjuvant therapy could, at least theoretically, downsize and downstage SRCG. However, survival benefit from perioperative treatment in diffuse tumors is not clear, as previously stated, and this would probably apply to SRCG and linitis plastica.

Molecular features

SRCG alterations include genes involved in the

immune response, metabolic and metastasis-associated pathways (Zhao et al., 2020). KRAS mutations have been identified in 12.3% of tumors (Lee et al., 2018b). 2.3% of cases demonstrate ALK rearrangements, so they can be potentially treated with ALK inhibitors, such as crizotinib (Zhao et al., 2016). HER2 amplification is less common than in other GC types (1.9%) (Woo et al., 2017). There is an increased risk of false HER2-positive diagnosis in SRCG, because the typical signet-ring cell morphology may simulate membranous accentuation of HER2 IHC cytoplasmic staining (HercepTest, Pathway) (Woo et al., 2017). 25% of SRCG show the CLDN18-ARHGAP26/6 fusion, which is associated with worse survival and no benefit from oxaliplatin/fluoropyrimidine-based chemotherapy (Zhang et al., 2018). In addition, early SRCG exhibits PIK3CA amplifications and a low rate of microsatellite instability; advanced SRCG tends to have programmed death-ligand 1 (PDL1) expression, which might be associated with peritoneal recurrence (Huang et al., 2020). According to recent reports, PDL1 and PD1 are expressed in 40.4% and 18% of SRCG, and 32.6% of tumors may lose at least 1 of the 4 mismatch repair proteins (Jin et al., 2017). Thus, immunotherapy can be a potential treatment for a significant number of SRCG patients.

C. Linitis plastica

Some authors have analyzed linitis plastica (LP) as a distinct subtype of GC, although it is generally grouped with diffuse-type in the Laurén classification (Burgain et al., 2016). As for mixed GC and SRCG, the term LP has been variably applied in the literature, and LP has been used as a synonym for SRCG. However, SRCG tumors can be pure SRCG, mucinous, mixed or they can involve diffusely and extensively the stomach wall, giving rise to LP.

LP was first described in 1779 as a rigid type of stomach with poor survival (Mastoraki et al., 2009). Later, it was thought to be a benign condition, leading to thickening of the gastric wall, with bands of filaments simulating linen ("linitis"). Finally, in 1953 it was confirmed to be a malignant condition with abundant fibrous tissue and scarce malignant cells (Agnes et al., 2017). This delay could be due to the difficulty of recognizing carcinoma cells in this entity.

LP constitutes about 6.3-25.3% of GC (Chang et al., 2017). As seen in SRCG, it is more frequent in women and in younger patients, and it can be related to familial GC (Blackham et al., 2016). LP can be primary (caused by GC) or secondary. Metastatic tumors, mainly lobular breast carcinomas, may involve gastric wall in a LP-like manner (Hamada et al., 2020).

Macroscopically, LP shows extensive infiltration of cancer cells into the gastric wall, causing a major segmental or diffuse thickening of the stomach and limiting gastric distensibility. Microscopically, scarce poorly cohesive and/or signet ring cells can be identified in an abundant fibrous stroma (Morgant et al., 2018).

Gastric specimens exhibit expanded submucosal connective tissue composed of mature and immature fibrous stroma, hypertrophy of the muscle layers and subserosal thickening; cancerous cells may be difficult to detect (Fig. 6).

Diagnosis

Currently, there is no standardized definition for LP, and several investigators have highlighted the need to reach a consensual definition for clinical and research purposes (Vivier-Chicoteau et al., 2020). LP has been described as the thickening of the gastric wall which affects at least one fourth, one third and two thirds of the stomach (Endo et al., 2012; Pedrazzani et al., 2012). Other authors described LP as circumferential involvement of more than one gastric area, or almost circumferential involvement of at least two gastric areas

(Agnes et al., 2017).

Preoperative diagnosis of LP can be challenging, because the concept of LP is based on autopsy findings and surgical specimens. Upper endoscopy is the gold standard for LP diagnosis, and it can show two types of alterations of the gastric mucosa: giant-fold and flat (Agnes et al., 2017). In the first type, mucosal foldings are enhanced and prominent; in the second type submucosal involvement leads to mucosal thickening or atrophy. However, biopsies may show normal mucosa or nonspecific inflammation in up to 30% of cases (Jung et al., 2016). Computed tomography studies may be helpful to confirm wall thickening, flattening of gastric mucosa and enhancement of the entire gastric wall (Burgain et al., 2016). Endoscopic ultrasound (EUS) and magnetic resonance imaging (MRI) can also assist in LP diagnosis, and a deep fine-needle aspiration guided by EUS can be performed (Kwee and Kwee, 2007; Shan et

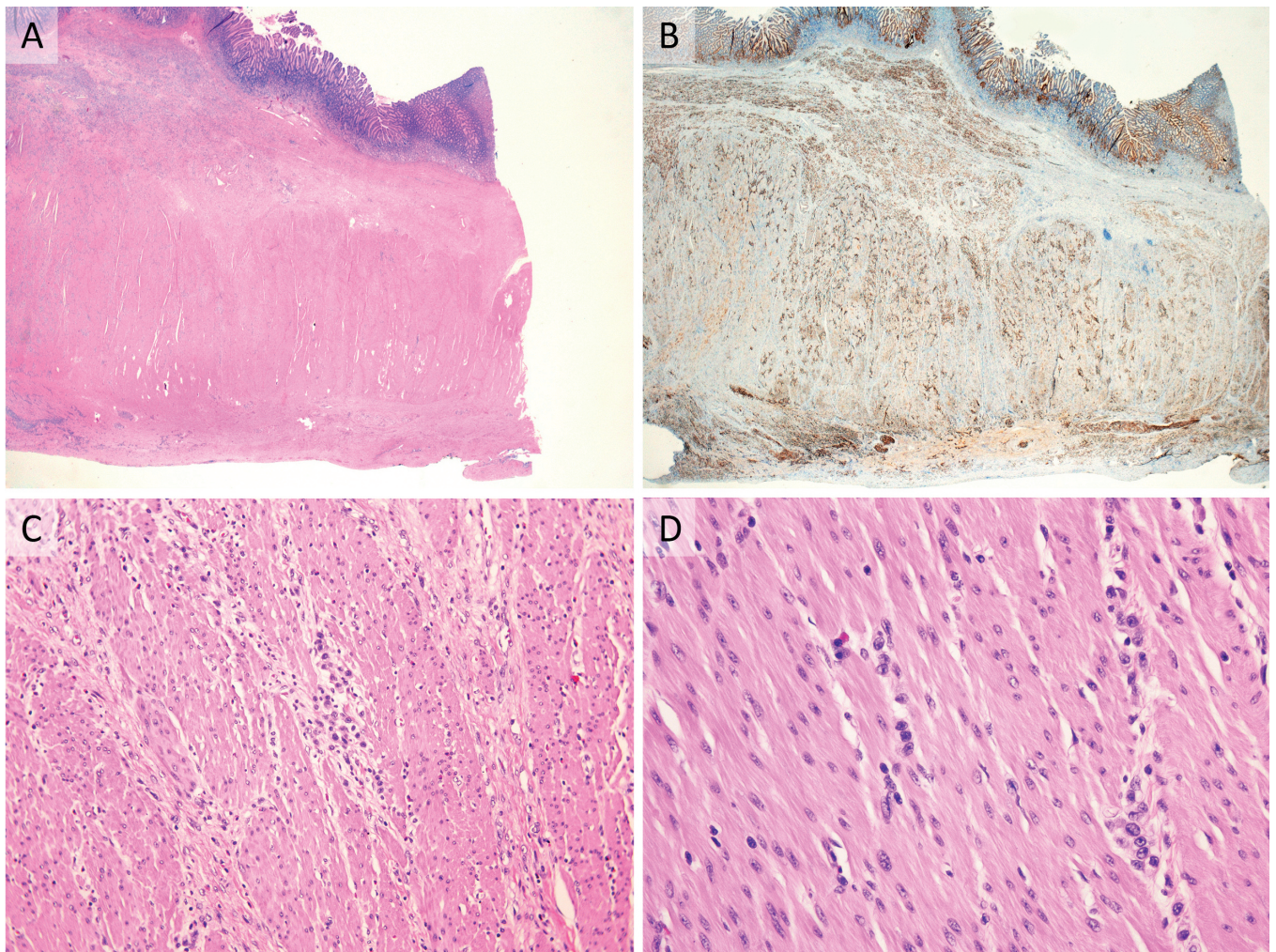


Fig. 6. Linitis plastica. Expanded gastric wall with prominent muscular layer and fibrous expansion of the submucosal connective tissue. Scarce poorly cohesive cells infiltrating the whole thickness of the gastric wall. Hematoxylin-eosin (A, C, D) and CKAE1-AE3 (B) staining.

al., 2013). In some cases, laparoscopic biopsies may be needed to reach a diagnosis.

One recent study analyzed 220 patients and developed a diagnostic score for LP diagnosis (Vivier-Chicoteau et al., 2020). This score included six parameters: large mucosal folds and/or parietal thickening on at least one location, diffuse gastric infiltration, gastric stenosis on endoscopic studies, circumferential thickening of at least one segment, thickening of the gastric wall predominant on the third hyperechoic layer on endoscopic ultrasound, and the identification of poorly cohesive and/or signet ring cells in endoscopic biopsies. LP score showed sensitivity and specificity rates of 94% and 88.7%, respectively.

Prognosis and management

In respect of disease outcomes, survival rates range between 2-12.5%, with median OS of 6-14 months (Chang et al., 2017). Some authors consider LP a distinct prognostic entity, but other investigators have observed similar prognosis between LP and non-LP patients. One previous study divided GC into intestinal, diffuse, and LP types. The authors did not find differences in survival between the intestinal and diffuse subtypes, but patients with LP showed poorer prognosis (Luu et al., 2017). LP was also associated with a higher TNM stage, and with higher rates of R1 resection, perineural infiltration and nodal metastasis. LP was an independent prognostic factor, and the investigators suggested that the survival differences reported between Laurén subtypes may be at least partially attributed to the presence of LP. However, other studies found no differences between survival rates of LP and non-LP patients, if LP tumors were optimally resected (Blackham et al., 2016). Thus, the survival differences between LP and non-LP patients may be related to the higher rate of suboptimal resections and lower rate of adjuvant therapy administration to LP patients.

There is controversy over the optimal management of LP patients. Due to their dismal prognosis, previous investigators rejected surgical treatment of LP (Jafferbhoy et al., 2013). However, recent studies have shown that the survival of LP patients after gastric resection is not different from patients with non-LP diffuse GC (Thompson et al., 2017). A subgroup of LP patients could benefit from gastrectomy and extensive lymphadenectomy, and they may reach survival rates similar to stage III non-LP resected patients (Blackham et al., 2016). In addition, staging laparoscopy and peritoneal washing cytology should be performed for patient selection, and the use of HIPEC in LP is being investigated (Yonemura et al., 2016).

Molecular features

Due to the characteristic features of LP, its molecular biology should be further analyzed. LP highlights the relevance of cell-stroma interaction, which can play an

important role in cancer progression. Cancer-associated fibroblasts are being studied (Terai et al., 2015), and several molecules may influence cell-stroma relationship, including hepatocyte growth factor (HGF), transforming growth factor- β (TGF- β) or fibroblast growth factor 7 (FGF7) (Yashiro and Hirakawa, 2010). Epithelial-to-mesenchymal transition (EMT) may also be crucial for LP development (Lamouille et al., 2014). EMT is defined as the transdifferentiation of epithelial cells into mesenchymal cells, and it was first described in chick embryos in the early 1980s. It was later related to wound healing and cancer aggressiveness. In EMT, epithelial cells lose their tight junctions and show reverse polarity, leading to an increase in cell motility and invasiveness. This process is mediated by several pathways and molecules, such as the TGF- β signaling pathway, SNAIL, zinc-finger E-box-binding (ZEB) or basic helix-loop-helix (bHLH) transcription factors. New molecular findings might lead to the expansion of targeted therapy in LP, and these molecules could also be useful biomarkers of cancer progression and therapeutic efficacy. As an example, specific inhibitors of TGF- β have been developed, and investigators have highlighted their potential benefit in cancer and fibrotic disorders (Caja et al., 2018).

Other histological classifications

The last World Health Organization (WHO) classification of tumors of the digestive system divides GC into four subtypes: tubular, papillary, mucinous and poorly cohesive (Bosman et al., 2019). This classification is purely morphological and it does not correlate well with GC histogenesis or biological behavior, limiting its value as a prognostic factor or as a surrogate for molecular classifications (Agnes et al., 2017).

The Japanese classification includes a histological classification equivalent to that published by the WHO (Sano and Kodera, 2011). However, according to this system, tumors can be further classified into medullary, scirrhous and intermediate type depending on the cancer stromal volume. LP can be equivalent to the Japanese scirrhous type. This protocol also stratifies GC into infiltrative a (INFa), INFb and INFc types according to the tumor infiltrative pattern into the surrounding tissues, and establishes a grading system for lymphatic (ly0, ly1, ly2, ly3) and venous invasion (v0, v1, v2, v3).

Grundmann et al. studied early and advanced GC and based their classification on 1) the cell type and architectural patterns and 2) depth and direction of invasion (Grundmann and Schlake, 1982). They classified GC depending on cell type into tubular, signet ring cell or anaplastic carcinomas. Depending on the type of invasion, tumors were focal, multifocal or superficial spread type. Finally, 8 levels of depth of invasion were defined.

Carneiro et. al divided GC into isolated-cell (6.6%), glandular (41.8%), solid (13.3%) and mixed carcinomas

(35.8%) (Carneiro, 1997). Mixed GC showed the worst prognosis. Ming et al. classified GC into expansive and infiltrative according to the pattern of growth at the invasive front (Ming, 1977). The Goseki classification assesses the degree of glandular differentiation and cytoplasmic mucus, and stratifies GC into four groups (grades I-IV) (Goseki et al., 1992). The last four classifications have not been widely adopted, but they may serve as a basis for future classifications.

Controversial aspects of histological classifications

As noted above, histology is an easy, cost-effective, rapid and widely available method for diagnosing and classifying GC. In addition, histological classifications can successfully reflect GC heterogeneity and molecular features, and they may play a central role in patient stratification in terms of prognosis and treatment individualization. After the development of molecular classifications of GC by TCGA and ACRG and the discovery of a correlation between the Laurén classification and molecular subtypes, Laurén subtype has regained importance in GC. Several authors have highlighted the need to include factors other than the TNM classification in clinical trials, such as Laurén classification, with the aim of homogenizing patient subgroups.

However, histological study of GC shows some limitations. First of all, variability exists in diagnostic criteria, due to some level of subjectiveness and the heterogeneity of the histological components of GC (Agnes et al., 2017). A 25-35% rate of disagreement between preoperative biopsy and resection specimen diagnosis has been recognized with respect to the Laurén classification, and 15% with regards to the WHO classification (Palli et al., 1991; Flucke et al., 2002). Moreover, interobserver disagreement ranges between 17-32% (Laurén classification) and 21-32% (WHO classification) in resection specimens. Second, GC subtyping is complicated by terminologic confusion and geographical variation. Laurén, WHO or stromal Japanese classifications have been used interchangeably in the literature. SRCG, LP and scirrhous tumors have not been well defined in some previous studies, and these terms are frequently used as synonyms. Moreover, LP was identified in autopsy studies, and a normalized preoperative definition should be developed for both resectable and non-resectable GC patients. Due to the relevance of GC subtypes and their impact on clinical practice and GC research, the terminology needs to be standardized and clearly defined to avoid misinterpretation. Third, some classifications correlate better with GC biology and patient outcomes than others. An optimal classification should reflect the morphological features of GC as well as its molecular heterogeneity and clinical behavior. Considering the available evidence, the translation of the morphological features of GC to the clinical practice and clinical trials is a cost-effective approach with a potentially huge

impact on patient homogenization and treatment selection in the new era of molecular medicine.

Tumor budding

Definition

Tumor budding (TB) is defined as the presence of single tumor cells or small clusters (up to 4 cells) in the invasive front of carcinomas. It was first described in the 1950 as “sprouting” at the leading edge of tumors and, since then, it has been recognized as a marker of cancer aggressiveness. In TB, finger-like projections arise from the primary tumor, invade into adjacent stroma, and some of them detach and form cell clusters (Fig. 7) (Dao et al., 2020). The term “tumor budding” was introduced in 1993 (Hase et al., 1993). In the last two decades, TB has been mainly studied in colorectal cancer (CRC), where it correlates with adverse outcomes. TB can be observed in other malignancies, such as esophageal cancer, pancreatic carcinoma, head and neck squamous cell cancer, cholangiocarcinoma, lung and breast tumors (Ulase et al., 2020). In some tumor types, TB has been identified as an independent risk factor (Koelzer et al., 2014).

The International Tumor Budding Consensus Conference (ITBCC) recognized TB as an independent prognosticator in CRC (Studer et al., 2021). In addition, TB is included in the guidelines for CRC diagnosis in Europe and Japan and it has been incorporated as a prognostic factor in the Union for the International Cancer Control (UICC) protocol for CRC (Watanabe et al., 2012; Von Karsa et al., 2013).

Assessment methods

TB definition has evolved over the years, and TB assessment methods vary widely between studies. Discordances include 1) staining techniques (hematoxylin-eosin or CKAE-AE3 IHC), 2) quantification versus subjective impression 3) area of assessment (hotspot or average) and 4) field size (Studer et al., 2021).

The first studies on TB applied qualitative methods and classified TB as high, moderate or low, but quantitative methods are currently widely used. Cytokeratin staining may facilitate the identification of tumor buds, but it does not increase the interobserver agreement (Puppa et al., 2012). Some authors have suggested that cytokeratin may be useful in cases with severe inflammatory infiltrates or brisk desmoplastic reaction at the invasive front, where TB can be difficult to evaluate (Studer et al., 2021). Previous research found an inverse association between TB and peritumoral inflammation (Max et al., 2016). As for the assessment area, tumor buds can be counted directly in a hotspot field, or they can be averaged over a certain number of high power fields or the entire invasive front. Field diameter varies depending on the microscope

model. For example, a 20x field may correspond to 0.785 mm² or 1.227 mm² (Studer et al., 2021). Thus, a normalization factor should be applied to correct these discrepancies.

One of the most used methods is the one proposed by Ueno et al. for CRC (Ueno et al., 2002). According to the authors, tumor buds should be counted in the area of maximal budding with a x200 (0.785 mm²) field. Cases are divided into high TB (≥ 5 tumor buds) and low TB.

Despite differences in assessing methods, reproducibility between studies is acceptable, and TB has been associated with clinicopathological features such as TNM stage, lymph node involvement, distant metastases, and poor prognosis in most previous research (Olsen et al., 2017).

ITBCC recommendations

In 2016, the ITBCC developed a consensus scoring system, based on the Ueno assessment method (Ueno et al., 2002). Hematoxylin-eosin sections are reviewed and single cells or clusters of less than 5 cells are identified. Tumor buds should be counted in the hotspot area in a x200 field (0.785 mm²). This area should be selected by screening at least 10 high power fields at the invasive front. TB is classified as low grade (0-4 buds), intermediate (5-9 buds), and high grade (≥ 10 buds). A three-tiered system was selected with the aim of improving patient subclassification.

The ITBCC also recommends to report the absolute number of tumor buds, because TB is a continuous

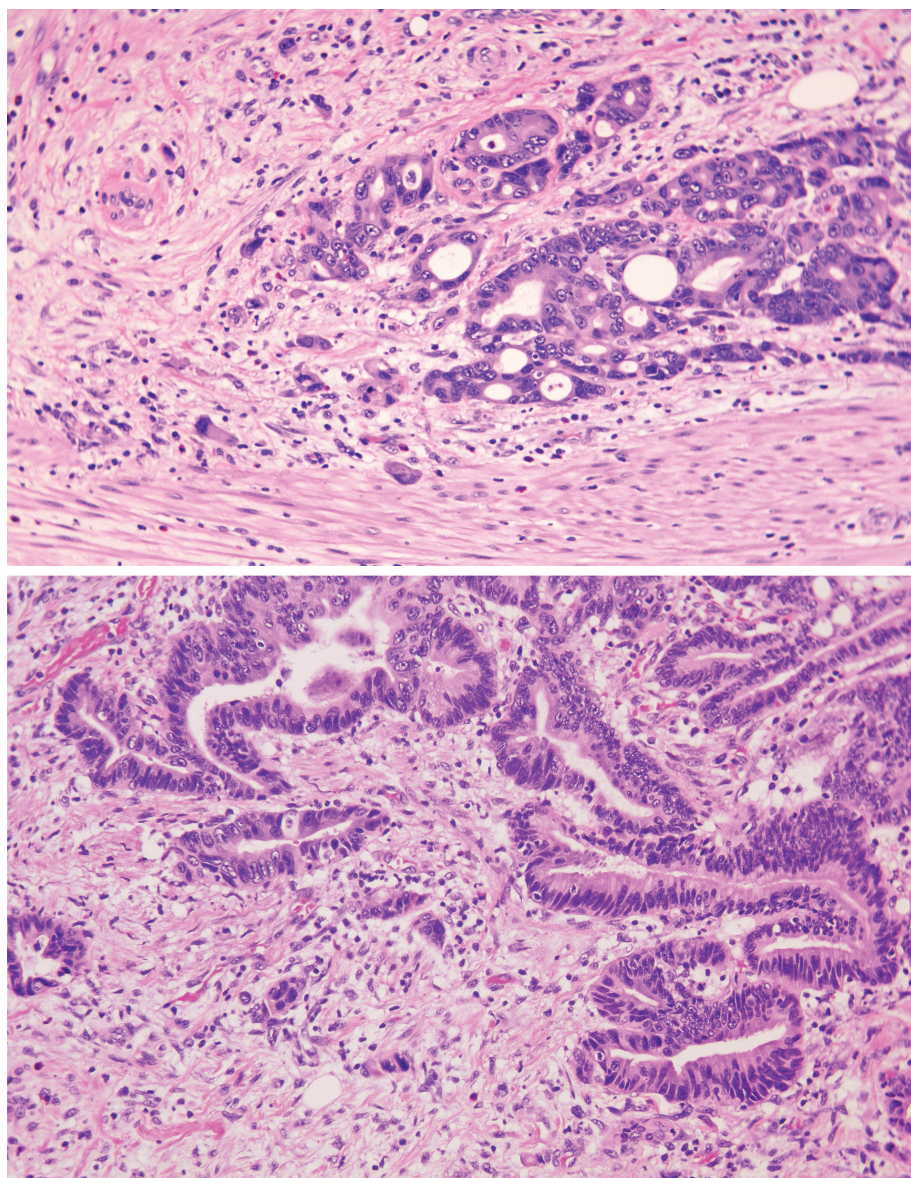


Fig. 7. Tumor budding. Intestinal-type tumors, invasive front. Detachment of single tumor cells and small clusters of cells. Hematoxylin-eosin staining.

variable with no upper limit (Studer et al., 2021). In addition, borderline cases are difficult to classify and a numerical value may allow for a more precise risk categorization.

Biological significance and molecular features

Tumor buds show downregulation of E-cadherin, nuclear translocation of beta-catenin and loss of cell polarity and cohesion (Ulase et al., 2020). Bud cells express mesenchymal markers and inducers of the EMT such as ZEB1, ZEB2 and tropomyosin related kinase B (TrkB, NTRK2). They exhibit upregulation of metalloproteinases, urokinase receptor and cathepsin, which lead to increased migration and stromal invasion (Pyke et al., 1995; Guzinska-Ustymowicz, 2006). Tumor buds may resist apoptosis via upregulation of RAS-kinase inhibitor protein and caspase-3 deficiency, and show low proliferative activity (Koelzer et al., 2013; Dawson et al., 2014). In fact, activating BRAF and KRAS mutations have been detected in high grade TB in CRC (Qi et al., 2020).

Based on these findings, most authors hypothesized that TB is a reflection of the EMT, and tumor bud cells are capable of migration due to reduced intercellular adhesion proteins, acquisition of a mesenchymal phenotype and degradation of the extracellular matrix (Berg and Schaeffer, 2018). Thus, TB confers a metastatic phenotype.

However, one study found that not all EMT-related proteins are involved in the TB process (Yamada et al., 2017). According to these results, TB regulation may be different than EMT, and the budding phenotype could be produced by another mechanism.

The current hypothesis is that tumor buds develop a “partial EMT” or “EMT-like” phenotype, because they show both adhesion and migration features (Guo et al., 2019). Tumor buds migrate in small groups, and some authors have observed that single-cell migration is rare, and it could be a two-dimensional artifact (Ulase et al., 2020).

Intratumoral budding

Apart from the leading edge of carcinomas, tumor buds can be identified in the intratumoral stroma. There is controversy over whether or not to assess intratumoral TB. Previous studies in CRC have demonstrated a correlation between intratumoral TB in biopsy material and peritumoral TB in resection specimens (Giger et al., 2012). The analysis of intratumoral budding may be useful in some contexts: sometimes it is difficult to determine the peritumoral area, many patients are diagnosed by small biopsies, and resection specimens cannot be studied in non-surgical cases (Qi et al., 2020).

Tumor budding in gastric cancer

Despite all previous evidence, only a few recent

studies have investigated the prognostic role of TB in GC. In our literature review, we have found 11 articles on this issue, and 6 of them were published in the last two years (Gabbert et al., 1992; Tanaka et al., 2014; Gulluoglu et al., 2015; Che et al., 2017; Olsen et al., 2017; Du et al., 2019; Kemi et al., 2019; Dao et al., 2020; Qi et al., 2020; Ulase et al., 2020; Zhang et al., 2020). The main features of these studies are summarized in Table 3.

In GC, high-grade TB has been identified in 31-63% of cases. It has been associated with female sex, tumor size, diffuse and mixed tumors, high grade, TNM stage, lymph node involvement, distant metastases, lymphovascular invasion, perineural infiltration, R1 resections and poor prognosis (Ulase et al., 2020). TB was an independent prognostic factor for OS in four studies.

In respect of the Laurén subtype, some authors have assessed TB only in intestinal GC, because they consider that diffuse tumors exhibit TB by definition (Berg and Schaeffer, 2018). 90.3% of diffuse GC demonstrate high TB according to another study (Ulase et al., 2020). Other authors found that the prognostic value of TB is limited or not significant in diffuse GC (Kemi et al., 2019). Thus, it is not clear whether to evaluate TB in carcinomas with primary dissociative growth pattern (Koelzer et al., 2014). Separate analysis should be performed to clarify the relationship between Laurén subtypes and TB, especially in diffuse GC.

One recent study evaluated the correlation between the immune microenvironment and TB in GC (Zhang et al., 2020). According to the authors, TB may be associated with a unique immune environment, recruiting special immune components. In this investigation, the numbers of tumor-infiltrating lymphocytes (TILs) and TB were inversely correlated: areas with intense TB showed fewer TILs than non-budding areas. Moreover, the immunologic profile changed from CD8>FOXP3>OX40>GrB T cells in non-budding regions to FOXP3 >CD8>OX40>GrB T cells in budding regions.

Another study analyzed intratumoral TB in GC (Qi et al., 2020). The investigators found a positive correlation between intratumoral and peritumoral TB. High intratumoral TB was associated with lymph node metastases and worse patient prognosis. Interestingly, cases with both intratumoral and peritumoral TB showed shorter OS than tumors with only peritumoral TB.

In respect of GC treatment, TB assessment may significantly influence treatment of early GC, due to the relationship between TB and lymph node metastases (Berg and Schaeffer, 2018). Early GC is defined as GC limited to the mucosa or submucosa, regardless of the lymph node status. TB has been identified as an independent predictive factor of lymph node metastases in early GC (Gulluoglu et al., 2015; Du et al., 2019). According to a recent study, lymphovascular invasion and TB are the main risk factors for lymph node metastasis in early GC, along with poorly cohesive

carcinomas, mixed tumors and female sex (Du et al., 2019). Therefore, TB could be included in the decision-making process for endoscopic GC treatment, together with other clinical and histological risk factors.

In addition, the role of multimodal therapy in T1N1 and T2N0 patients is not clear, and some authors have suggested that TB can be an indicator of adjuvant therapy in stage IB/II patients after surgical resection (Koelzer et al., 2014).

In advanced GC, TB determines patient prognosis, and it may add useful information for targeted treatment planning. Previous studies found an inverse correlation between TB and HER2/MSI status, and a positive

correlation with MET status (Ulase et al., 2020). TB may also be associated with chemotherapy resistance via TrkB expression (Kupferman et al., 2010; Fujikawa et al., 2012).

Future perspectives

TB can be further standardized by automatized methods. A recent review analyzed the current advances in the automated detection of TB, and found 12 articles on this issue (Studer et al., 2021). Most methods require the manual selection of a region of interest, and they are based on IHC-stained slides. Only one method proposed

Table 3. Tumor budding in gastric cancer: previous studies.

Study	Method	Cases N Laurén Stages	Low/high TB (%)	Clinicopathological features (univariate analysis)	Prognosis Multivariate analysis
Gabbert et al., 1992	Select hotspot Semi-quantitative HG ^a : ≥5 buds	445 I+D ^b I-IV	48.3/51.7	Tumor grade, T stage, N stage, positive circumferential margin	HG TB ^c : lower OS ^d (significant) Multivariate: independent PFe for OS
Gulluoglu et al., 2015	Select hotspot at 100x magnification Qualitative assessment at 400x	126 I+D Early	69/31	Analysis of the association between TB and clinicopathological variables not performed	OS or DFS ^f depending on TB: not assessed Multivariate: TB is an independent PF for lymph node metastases
Tanaka, 2016	CKAE1-AE3. Select hotspot at 100x magnification Count 3 400x fields HG: ROC analysis	153 Int. I-IV	40/60	Size, macroscopy, tumor grade, lymphovascular invasion, T stage, N stage, distant metastases, TrkB expression	HG TB: lower OS with intestinal tumors (significant) Multivariate: not independent
Olsen et al., 2017	Select two slides Select 5 hotspots in each slide (total: 10) Count 10 200x fields HG: median	104 Int. I-IV	37/63	Tumor grade, lymphovascular invasion, perineural infiltration, T stage, N stage, recurrence	OS or DFS depending on TB: not assessed Multivariate: not independent for DFS
Che et al., 2017	Select hotspot at 100x magnification Count 10 400x fields HG: ≥5 buds	296 I+D I-IV	51/49	Analysis of the association between TB and clinicopathological variables not performed	HG TB: lower OS (significant) Multivariate: high TB is an independent PF for OS
Kemi et al., 2019	Select hotspot with low magnification Count one 200x field (0.398 mm ²) and multiply buds by 1.97 (0.785 mm ²) HG: ≥10 buds	583 I+D I-IV	44.9/55.1	Age, female sex, Laurén, tumor grade, TNM stage, R1 resections	HG TB: lower OS (significant) Subgroup analysis: lower OS in intestinal GC (significant). No statistical differences in OS in diffuse GC
Du et al., 2019	Qualitative assessment at 200x	621 I+D Early	59.3/40.7	Analysis of the association between TB and clinicopathological variables not performed	OS or DFS depending on TB: not assessed Multivariate: TB is an independent PF for lymph node metastases
Ulase et al., 2020	ITBCC	456 I+D I-IV	48/52	Sex, Laurén, tumor grade, T stage, N stage, distant metastases, lymphatic invasion, perineural infiltration, HER2, MET, MSI status	HG TB: lower OS and DFS (significant) Multivariate: not independent
Zhang et al., 2020	Select hotspot at 100x magnification Count 5 200x fields HG: median	147 I+D I-IV	NS ^g	Tumor size, Laurén, N stage, TNM stage	HG TB: lower OS (significant) Multivariate: high TB was an independent PF for OS
Dao et al., 2020	ITBCC ^h	109 I+D I-IV	54.1/45.9	Laurén, tumor grade, lymphovascular invasion, perineural infiltration, lymph node status, recurrence	HG TB: lower OS and DFS (significant) Multivariate: high TB is an independent PF for both DFS and OS
Qi et al., 2020	Select hotspot Count 5 400x fields HG: median	153 Int. I-IV	NS	PT ⁱ TB: Age, sex, tumor location, lymph node involvement IT ^j TB: Tumor size, tumor grade, lymph node involvement, pTNM	HG IT TB and HG PT TB: lower OS Multivariate: both IT TB and HG PT TG are independent PF for OS

^aHG, high grade; ^bLaurén subtype, I (intestinal), D (diffuse); ^cTB, tumor budding; ^dOS, overall survival; ^ePF, prognostic factor; ^fDFS, disease-free survival; ^gNS, not specified; ^hITBCC, International Tumour Budding Consensus Conference. Bd1 (0-4 buds), Bd2 (5-9 buds), Bd3 (≥10 buds). Low grade: Bd1+Bd2. High grade: Bd3; ⁱPT, peritumoral; ^jIT, intratumoral.

an end-to-end solution, and another method assessed hematoxylin and eosin-stained slides. Automated systems showed accuracy and sensitivity rates of 97.7% and 93.4%. Hematoxylin and eosin-based automated methods showed lower sensitivity rates (72%). Cutoffs should also be modified depending on the automated system used.

As previously stated, further research should evaluate the role of TB depending on Laurén classification, specimen type (biopsy or resection), tumor stage and TB location (intratumoral or peritumoral), and its relationship with peritumoral inflammation in GC.

Inflammatory infiltration

The role of the immune microenvironment and predictors of immunotherapeutic (IT) response are currently being analyzed in several tumor types. Immune checkpoint inhibition is the most known IT approach. IT drugs target inhibitory receptors located on the surface of T lymphocytes (mainly cytotoxic T-lymphocyte associated protein 4 -CTLA4- or PD1). This blockage allows the T-cell to act against tumor cells, and prevents tumor evasion from immunosurveillance (Hamanishi et al., 2016). As seen in other tumors, a subset of GC patients responds to IT (10-20%) (Kim et al., 2019). IT efficacy is modulated by the tumor microenvironment and immunogenicity. In respect of GC immunogenicity, tumors expressing PDL1 (combined positive score ≥ 1) or with MSI phenotype are treated with IT agents, such as Pembrolizumab (anti-PD1) (Le et al., 2015; Fuchs et al., 2018). Objective response rates have been modest, and some investigators have pointed out the need to improve patient selection and to identify better predictors of IT response (Zaanan and Taieb, 2019). In respect of MSI GC, microsatellites are regions distributed randomly throughout the genome, composed of short repetitive DNA sequences (Cortes-Ciriano et al., 2017). MSI tumors occur due to germline mutations or sporadic epigenetic silencing affecting the mismatch repair system genes; these alterations lead to failure in repairing insertions or deletions in the microsatellite regions during replication. MSI GC responds to IT due to its high mutational load, which creates neoantigens (Xiao and Freeman, 2015). These tumors show frequent amplifications of receptor tyrosine kinase and cell-cycle genes, and mutations in genes such as PIK3CA, TP53, ARID1a or KRAS (Cancer Genome Atlas Research Network, 2014). As previously noted, MSI tumors are usually intestinal-type tumors according to the Laurén classification. MSI can be detected by IHC staining of mismatch repair proteins and/or polymerase chain reaction (PCR) assays. Apart from MSI GC, the EBV molecular subtype of GC has also been recognized as responsive to IT drugs (Rodríguez et al., 2020). EBV GC constitutes 8.2% of GC patients, it occurs in young males and is located in the gastric fundus or body; EBV tumors can be intestinal or diffuse (Murphy et al., 2009).

TCGA investigators found that EBV GC is related to PDL1 and PDL2 overexpression, PIK3CA mutation and ERBB2/JAK2 amplification (Cancer Genome Atlas Research Network, 2014). It also shows overexpression of genes involved in the immune pathways and higher levels of DNA methylation. These tumors are identified by in situ hybridization methods. Finally, PDL1 expression can be measured by IHC: different tests are performed for each specific IT drug (Kim et al., 2017).

Lymphocytes

The functions of tumor microenvironment and inflammatory infiltrates in GC have been less extensively studied. Tumor-infiltrating lymphocytes (TILs) are the most important inflammatory cells of the cancer-immune interaction (Lee et al., 2018c). Tumors can be divided into “hot” (T-cell-inflamed) and “cold” (non-inflamed) (Woo et al., 2015). In “hot” GC, cancer cells are surrounded by T cells, B cells, myeloid leukocytes, natural killer cells, macrophages and dendritic cells. “Hot” tumors are recognized as immunologically responsive (Salgado et al., 2015).

Regarding tumor-associated inflammation, MSI or EBV GC are the most studied GC types. They exhibit an extensive immune infiltration and higher rates of PDL1 expression (Ma et al., 2016a; de Rosa et al., 2018). They have been associated with higher numbers of infiltrating CD8+ T cells, and a CD8:CD4 ratio of >1 (Van Beek et al., 2006).

One recent study divided GC into intestinal, diffuse, MSI and EBV (Kim et al., 2019). The investigators found that the most frequent tumor-infiltrating leukocytes were CD8+ T lymphocytes and CD68+ macrophages. As previously reported, EBV and MSI tumors exhibited the highest immune infiltration. Interestingly, the type and extent of the immune infiltration depended on Laurén subtype: intestinal tumors contained moderate T cells compared with MSI and EBV types, but relatively more macrophages. Intestinal-type associated macrophages showed inhibitory marker expression. Diffuse GC presented an “immune-desert phenotype” with fewer immune cells, and largely lacked macrophages. In respect of PDL1, it was expressed more frequently in EBV and MSI tumors (70%), followed by intestinal (36%) and diffuse GC (17%). On the contrary, another recent series indicated that diffuse tumors presented more CD8+ T cells, but they were less functional; infiltration by CD8+ cells was related to better outcomes only in patients with intestinal-type tumors (Li et al., 2020). A positive correlation between CD8 positivity and PDL1 expression has been observed in GC (Thompson et al., 2016).

The relationship between lymphocytic infiltration and GC prognosis remains controversial. Some authors have found that CD8+ T cell density and higher PDL1 expression in tumor cells correlate with poorer OS and DFS in GC (Thompson et al., 2016). Higher numbers of CD8+ T cells and TILs have been linked to better OS in

another study (Yu et al., 2018). The prognostic significance of tumor infiltration by CD4+ T cells or PDL1 expression is also not clear (de Rosa et al., 2018; Zhang et al., 2019).

Three meta-analyses included research on TILs and GC prognosis (Zheng et al., 2017; Lee et al., 2018c; Zhang et al., 2019). In one of them, the amount of CD3+ TILs was recognized as the strongest prognostic marker for OS in GC: the presence of CD3+ lymphocytes indicated better OS (HR: 0.52) (Zheng et al., 2017). In another meta-analysis, CD8+ TILs were significantly related to both DFS and OS (Lee et al., 2018c).

As previously discussed, one study evaluated the association between TB and immune infiltration in GC (Zhang et al., 2020). The authors found that the activated cytotoxic T lymphocytes (GrB + T cells) were highly related to patient prognosis, but the number of cytotoxic T-lymphocytes (CD8+ T cells) may not be so sensitive. Thus, the recognition of different subtypes of CD8+ T cells could add important prognostic information in cancer patients.

An immune microenvironment classification based on PDL1 status and the identification of TILs has been currently applied to GC (Cho et al., 2018). Tumors were grouped depending on PDL1 (combined positive score) and CD8 expression as follows: type 1 (PDL1+/TIL+, adaptive immune resistance), type 2 (PDL1-/TIL-, immune ignorance), type 3 (PDL1+/TIL-, intrinsic oncogenic induction of PDL1) and type 4 (PDL1-/TIL+, role of other suppressors in promoting immune tolerance). Type 1 tumors (40%) were composed mainly by EBV and MSI tumors, and showed the best prognosis. Type 2 tumors (30%) exhibited the worst survival. Type 3 and 4 tumors constituted 10% and 20% of GC, showed an intermediate prognosis, and included some EBV and MSI tumors.

Finally, immunological profiles for GC have also been developed based on sequencing techniques, and they seem to correlate with the density of tumor-infiltrating cells and previous molecular classifications (Sato et al., 2020).

Natural killer cells

Natural killer (NK) cells are cytotoxic lymphoid cells (CD57+) which secrete cytokines and cytolytic granules, and eliminate target cells (Ben-Shmuel et al., 2020). Thus, the promotion of NK functions can be effective in eradicating cancer cells. NK-based therapies are a promising immunotherapy, and several clinical trials have detected survival benefit from “pro-NK” agents, mainly in hematopoietic tumors. In GC, the number of apoptotic NK cells is higher than in normal controls, and it correlates with tumor progression (Saito et al., 2013). Moreover, higher density of NK cell infiltration is related to prolonged survival rates (Cui et al., 2015). Tumors with NK infiltration show reduced size, less lymph node metastases, less suboptimal resections and better OS (Ishigami et al., 2000).

Peritumoral infiltration

Crohn's like lymphoid reaction (CLR) is a marker of peritumoral lymphocyte activity, and it has been identified as a prognosticator in various tumor types (Maoz et al., 2019). It reflects tumor immunogenicity, and it is related to MSI in colorectal cancer. CLR is defined as the presence of aggregates of lymphocytes at the invasive front of solid tumors. As far as we know, only one study has analyzed the role of CLR in GC (Omura et al., 2020). In this study, the size of the peritumoral lymphoid clusters showed prognostic value. Larger CLR was associated with better OS and DFS, and it was also related to better nutritional status (assessed by body mass index, body composition status and Onodera's prognostic nutritional index).

Macrophages

Tumor-associated macrophages (TAMs) originate from circulating monocytes and they are the most abundant immune population in the tumor microenvironment (Kim et al., 2015). Depending on their interaction with local factors, TAMs exhibit two different phenotypes: type M1 (classically activated) and M2 (alternatively activated) (Biswas et al., 2013). Type M1 TAMs are triggered by T-helper type 1 (Th1) cytokines, microbial stimuli and tumor necrosis factor α (TNF- α). They secrete cytokines, chemokines and reactive nitrogen/oxygen species, and activate the inflammatory response and several antitumor processes. Type M2 TAMs are induced by Th2 cytokines (IL10, IL4, IL13), and they are involved in tissue remodeling, angiogenesis, tumor cell activation and anti-inflammatory response. TAMs seem to express the M2 phenotype more frequently than the M1 phenotype. Thus, they exert mainly protumorigenic functions. The prognostic value of TAMs in human cancer has been debated, and a relationship between TAMs and poorer survival has been observed in several tumor types such as liver, lung, prostate or breast cancer (Yin et al., 2017). However, controversy exists over the role of TAMs in GC. Some authors have associated TAMs with angiogenesis, lymphangiogenesis and poorer survival (Ishigami et al., 2003; Wu et al., 2012), but other investigators found no relationship between TAMs and patient outcomes (Zhang et al., 2015). Moreover, TAMs were an independent good prognostic factor in GC in a previous study (Wang et al., 2011). A current meta-analysis including 19 articles and 2242 patients found that CD68+ TAM infiltration has neutral effects on OS (Yin et al., 2017). These studies used CD68 as a marker for TAMs, but this marker may not reflect adequately the phenotypic variability of TAMs, because both M1 and M2 types are CD68+. Other authors have proposed CD163 as a marker of M2 macrophages, although it can also stain other cell subsets, such as dendritic cells (Kim et al., 2015). Low density of CD68+ or CD163+ TAMs in four combined areas of MSI-high GC has been

associated with low-grade histology and intestinal-type tumors. However, the presence of abundant CD163+ TAMs has been positively correlated to the number of TILs, and it may be an independent prognostic factor for better DFS in GC.

Neutrophils

Neutrophils are the most abundant circulating inflammatory cells, and the first responders of the innate immune response (Powell and Huttenlocher, 2016). These cells release cytokines and chemokines, and they are also involved in the antigen presentation process. In the last years, they have been recognized as components of the tumor microenvironment. As seen in TAMs, tumor associated neutrophils (TANs) can be classified into two groups according to their phenotype (Masucci et al., 2019). N1 TANs are short-living, cytotoxic cells with a mature phenotype. N2 TANs show less immune-stimulating activity, and they are long-living, low cytotoxic cells with a mature phenotype, which promote angiogenesis, metastases and immunosuppression. It has been suggested that the tumor microenvironment regulates TAN phenotype. Currently, there are no specific markers to identify TAN N1 and N2 neutrophils, although some morphologic clues have been described (Hagerling and Werb, 2016). TANs have been linked to tumor progression and poor clinical outcomes in cancer patients (Mizuno et al., 2019). In respect of GC, one in vitro study indicated that myeloid activated cells promote migration and invasion in gastric cancer cells, via activation of the ERK pathway and epithelial-mesenchymal transition (Zhang et al., 2017). Neutrophil infiltration has been identified as an independent risk factor for lymph node metastases in early GC (Wang et al., 2020b).

Mast cells

Mast cells constitute a heterogeneous population of cells, widely distributed throughout human tissues, which play important roles in many types of innate and adaptive immune responses (da Silva et al., 2014). More recently, they have been recognized as mediators in several processes, such as the defense against infectious organisms, angiogenesis, wound healing, metabolic and autoimmune disorders and cancer development. These cells can secrete cytokines, growth factors and mitogens, including TNF, interleukins, EGFR, FGF2 or VEGF, which contribute to their protumorigenic role. The function of each molecule has been extensively reviewed (Mukai et al., 2018). Mast cells are increased in human cancer, and their effect on cancer progression may depend on their microlocalization and tumor type and stage (Varricchi et al., 2017). In GC, mast cells have been related to angiogenesis, lymph node metastases and patient survival (Sammarco et al., 2019). Interestingly, tumor-infiltrating mast cells show high rates of PDL1 expression and might play a role in the immune

tolerance in GC, suppressing T-cell immunity and promoting GC growth (Lv et al., 2019). Mast cell functions can be inhibited therapeutically via promotion of apoptosis or blockage of degranulation, so they are potential therapeutic targets in cancer patients (Varricchi et al., 2017).

Limitations

There is a major limitation of the tumor microenvironment approach, as compared with other prognostic factors discussed in the present review. Conventional hematoxylin and eosin-stained slides can be sufficient to select cases of highly inflamed GC. However, a limited panel of IHC markers should be applied to better characterize the type of immune infiltration. Based on previous results, at least CD3, CD8, CD68 and CD163 should be performed. Fortunately, these markers are widely available and validated in laboratories, and they can add useful information in tumors with high density of TILs. On the other hand, PDL1 IHC, as IHC evaluation of HER2 status, has been increasingly incorporated in laboratories in recent years; it may further expand the characterization of the immune landscape of GC.

Conclusions

Histology is a cheap and widely available method with prognostic value in gastric cancer patients. In the era of molecular medicine, histological features are acquiring new roles as reflectors of the genotype-phenotype correlation, and they can be useful for patient stratification and personalized treatment. In particular, Laurén subtypes have been associated with the new molecular classifications of GC, and several authors have highlighted the need to include this classification as a criteria for patient grouping in clinical trials, because Laurén subtypes respond differently to GC therapies. TB has been recently studied in GC, and most investigators found a relationship between high-grade TB and poor prognosis. Moreover, molecular features of TB are being investigated, and primary results show that TB may influence treatment selection and response to certain drugs in solid tumors. More studies on TB and GC should be performed to increase the available evidence. Finally, the role of the tumor microenvironment in cancer has gained importance in the last years, due to the increasing impact of immunotherapy in cancer research and treatment. In GC, lymphocytic infiltration is associated with certain molecular features (MSI or EBV types) and higher expression of PDL1. Neutrophils and macrophages mainly show protumorigenic effects, and NK-based drugs could be active against tumor growth. However, the assessment of inflammatory infiltration has not been standardized, and inflammatory cell subsets are still being characterized. Moreover, there is controversy over the prognostic role of inflammatory cells in cancer, and more research should be conducted

to clarify their potential effects on carcinogenesis, patient outcomes and treatment response in several tumor types.

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Research involving human participants and/or animals. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent. Retrospective study; for this type of study formal consent is not required.

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