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A consolidated working classification of gastric cancer for histopathologists (Review)

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Abstract. Gastric cancer (GC) remains a disease with poor prognosis despite increasing availability of more effective targeted treatment. This may be in part due to the difficulty in selecting patients for appropriate treatment. Conventional taxonomic classifications of GC are ill-suited to make full use of recent advances in personalised therapy. In the past decade a number of molecular classifications have been proposed to address this; however, to date, there has been little implementation in the diagnostic routine. The lack of harmonisation between these classifications, the complexity and unavailability of some of the tests required plus the demands on time and resources, all contribute to poor uptake in the diagnostic routine. In the present study, these classifications were reviewed and an inclusive working classification that includes their main points, focuses on prognosis and treatment options and can be delivered using four on-slide tests (*in situ* hybridization for Epstein-Barr encoding region and immunohistochemistry for mismatch repair, E-cadherin and p53) is proposed. These tests can be performed on paraffin-embedded tissue and could be available in the majority of histopathology laboratories. The proposed classification also includes reflex testing for specific biomarkers relevant to treatment selection.

Contents

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1. Introduction

Gastric cancer (GC) is an aggressive disease, numerous patients are diagnosed at advanced stages and some are inoperable (1,2). It consists of several subtypes; their relative incidence is influenced by genetic and environmental factors, thus, prevalence of each subtype may vary significantly in different populations (3). Conventional treatment is of limited success (4-8). Recent advances in personalised treatment improve outcomes but, for this to be effective, distinct subtypes need to be recognised. Different morphological and molecular subtypes have been highlighted by numerous classifications, but no unifying classification is currently in use. In the present study, the main classifications were reviewed in order to provide a workable scheme that includes the important elements of each, so they can be delivered with on-slide tests in the diagnostic routine and prognostic and predictive data can be provided. It is important that a working molecular classification is structured to include present companion diagnostic biomarkers necessary for selection for biological therapies. Since new biological therapies will inevitably emerge, this classification needs to be able to accommodate new companion diagnostics. Finally, for a working classification to have a significaticant impact on patients outcomes worldwide, it needs to be implementable in histopathology departments using available resources.

2. The Laurén Classification

The Finnish pathologist Pekka Laurén first recognised GC as a heterogenous group of diseases and, in 1965, published his reductive histological classification consisting of only three subtypes that associated with the biology of the disease (9).

He described two main types: intestinal and diffuse, both with no direct counterpart in previous morphologic classifications. As a general principle, the intestinal type had recognisable morphological counterparts in colorectal cancer whilst the diffuse type did not. The intestinal type is characterised by cells resembling those found in the small intestine. It tends to be slower growing and less aggressive than the diffuse type, which is characterised by cells that infiltrate more widely and are less organised. Laurén also recognised a minor group (~15% of cases) that did not sit within these two types and later termed 'atypical' or 'indeterminate' (9). In 1995, Carneiro and colleagues divided Laurén's indeterminate category into mixed tumours (having at least 5% of both intestinal and diffuse components) and solid tumours (10).

There was little understanding of the molecular and biological significance of loss of cell-to-cell adhesion in 1965. More recently, this has been linked with epithelial-to-mesenchymal transition (EMT). Nevertheless, Laurén recognised that tumours with loss of cell-to-cell adhesion represented a separate entity. Authors' opinion suggest that loss of cell-to-cell adhesion (11,12) is the linchpin of the Laurén classification.

3. The World Health Organisation (WHO) classification

This classification recognises molecular subgroups (see below, intrinsic classifications) but even in its latest (2019) edition, remains fixed on taxonomy and has dozens of different morphological types. It describes five main subtypes of adenocarcinoma: i) tubular (the most common), ii) papillary, iii) poorly cohesive (this includes signet ring cell and other subtypes), iv) mucinous and v) mixed (13). Adenocarcinoma represents 95% of all malignant epithelial tumours of the stomach (14). The World Health Organisation (WHO) classification does not provide sufficient details to drive personalised treatment and, notably, the Laurén classification continues being described, even in the current WHO publication, due to its direct relevance for prognosis and treatment.

4. Intrinsic classifications

The Laurén and WHO classifications are focused on the morphology of the tumour cell compartment. ~20 years ago, the intrinsic properties of the tissue (rather than of the tumour cells alone) were investigated with a view to provide important clues for tumour classification, tumour behaviour and response to treatment. Such classifications represented a significant step forward in improving outcomes for patients with GC. Since then, the intrinsic properties of tumours have been scrutinised by genomics, transcriptomics and proteomics of the whole tumour tissue and intrinsic classifications have been proposed for numerous tumour types, including ovarian, colon, breast, endometrium and lung (15-19).

The two major intrinsic molecular classifications for GC are those of The Cancer Genome Atlas and the Asian Cancer Research Group. Both are large scale, multi-institutional studies that characterise the changes that occur in GC using multi-omics techniques. Both aim at understanding the biology of the disease to identify new therapeutic targets (20-22). The Cancer Genome Atlas (TCGA) and the ACRG classifications are briefly described below.

TCGA intrinsic classification. In 2014, TCGA proposed a classification of gastric carcinoma based on the genetic and molecular characteristics of the tumour (20). It was developed by TCGA research network as part of a large-scale effort to understand the underlying basis of gastric carcinoma. Tissues from 295 cases of primary gastric carcinoma with no prior chemotherapy or radiotherapy were studied using six different molecular platforms, including single nucleotide polymorphism array, somatic copy-number analysis, whole-exome sequencing, mRNA sequencing, miRNA sequencing, array-based DNA methylation profiling and reverse-phase protein arrays. A subset (77%) was also tested by whole genome sequencing (Next-generation sequencing).

This work resulted in the grouping of GC into four major types: i) GC associated with Epstein-Barr virus (EBV), ii) GC with mismatch repair deficiency (dMMR), iii) genomically stable (GS) GC and iv) GC with chromosome instability (CIN).

GC-EBV(+). GC-EBV(+) (9%) is more common in younger individuals, predominately male patients and has an improved prognosis compared with other subtypes. These tumours are mainly in fundus or body (62%), have hypermethylation of CDKN2A ($p16^{INK4a}$) promoter, have the highest rate of phosphoinosytol-3 kinase (PIK3CA) mutations (80%) and overexpress programmed death-ligand (PD-L)1/2 (15%) due to amplification at 9p24.1, a locus containing genes encoding for JAK2, PD-L1 and PD-L2.

GC-dMMR. GC-dMMR (22%) is typically more aggressive than other subtypes and affects older patients (median age, 72 years). It is characterised by hypermethylation of MLH1 promoter. Mutations in PIK3CA are also common in this subtype (42%).

GC-GS. GC-GS (20%) is characterised by low levels of genetic instability and tends to be slower-growing. It is predominantly of diffuse histology (73%) and is associated with CDH1 (E-cadherin) mutations (37%), RHOA mutations (30%) as well as CLDN18-ARHGAP (Claudin-18) rearrangements (30%). The latter two mutations are mutually exclusive; they affect key molecules in cell-to-cell adhesion and are probably responsible for the diffuse growth pattern.

GC-CIN. GC-CIN (50%) subtype is characterised by amplification of receptor tyrosine kinases, has a high percentage of TP53 mutations (73% of CIN tumours) and corresponds to the intestinal type of Laurén. Numerous genes are affected in this subtype, including VEGFA (7%), ERBB2 (24%), ERBB3 (8%), ERBB1 (10%), FGFR2 (8%) as well as c-Met (8%).

ACRG intrinsic classification. There are significant differences in GC arising in the Asian population, possibly related to a combination of genetic and environmental factors. In 2014, ACRG published a different molecular classification for GC. Initially, they performed whole genome sequencing on tissue from 49 gastric tumours (22), and later added gene expression profiling, genome-wide copy number, microarrays and targeted gene sequencing from a further 251 cases (21).

ACRG divide GC into MMR-proficient (pMMR) and MMR-deficient (dMMR) types. The pMMR GC is further divided into three subtypes. Their proposed four molecular

subtypes have some overlap with TCGA groups and are GC-dMMR, GC with EMT (GC-EMT), CG with intact p53 (GC-p53wt) and GC with functional loss of p53 due to mutation (GC-p53m).

GC-dMMR. The GC-dMMR (23%) group contains tumours that are mainly located in the antrum, are usually diagnosed at early stages (clinical stage I/II), have the lowest frequency of recurrence and, when recurrence occurs, this is usually in liver. These tumours are predominantly intestinal type (60%) and have the best prognosis. In this group, there are GC with mutations in KRAS (23%), PI3K-PTEN-mTOR (42%), ALK (16%) and ARID1A (44.2%). This subtype has an overlap with the GC-dMMR of TCGA classification.

GC-EMT. The GC-EMT (15%) subtype is characterised by loss of CDH1 (E-cadherin) and is predominantly observed in young patients. These tumours correspond to the diffuse type of Laurén, they are diagnosed at late stages (80% are clinical stages III/IV) and therefore have worse prognosis with high recurrence rates (mainly in peritoneal cavity). This subtype has the lowest number of mutational events and has overlaps with the GC-GS of TCGA classification.

GC-p53wt. The GC-p53wt (26%) group is found more frequently in male patients, is mostly of the Laurén's intestinal type and has intermediate prognosis. Numerous of these patients are diagnosed in early stages (clinical I/II). This subtype has a higher mutation rate in APC, ARID1A, KRAS, PIK3CA and SMAD4. In addition, this group contains the highest proportion of integrated EBV and therefore may overlap with the GC-EBV(+) of TCGA classification.

GC-p53m. The GC-p53m (36%) subtype is identified more frequently in male patients and is of Laurén's intestinal type. It is diagnosed at advanced stages and has intermediate prognosis. This group is characterised by high prevalence (60%) of TP53 mutation, is associated with amplification in ERBB2, ERBB1, CCNE1 and CCND1 genes and may overlap with the GC-CIN of TCGA classification.

It is recognised that while there is some overlap with TCGA, a major difference is that the ACRG lacks a category that relies solely on EBV status, whilst TCGA does not have a category reliant solely on p53 status.

5. Singapore-Duke classification

Numerous other classifications of GC have been proposed. Some focus on clinical or surgical parameters, which have less relevance to the pathologist, whilst others target the cellular biology of GC using *in vitro* models. It is worth describing one such classification, the Singapore-Duke classification, which highlights the current gap in knowledge of the intrinsic classifications. This is focused on molecular *in vitro* studies and is based on different biological properties and response to chemotherapy and targeted therapy (23). It identifies three GC subtypes

i) Mesenchymal subtype. The mesenchymal GC has highly activated EMT molecular pathways, low rates of TP53 mutation and low level of CDH1 (E-cadherin) expression. This subtype has cancer stem cell-like properties, corresponds to the Laurén's diffuse type and is more sensitive to PIK3CA and mTOR inhibitors.

ii) Proliferative subtype. The proliferative subtype has high rates of TP53 mutation, more extensive gene amplification, high levels of genomic instability and DNA hypomethylation and corresponds to Laurén's intestinal type.

iii) Metabolic subtype. The metabolic type has low rates of TP53 mutation, expresses genes characteristic of normal gastric mucosa and has no intuitive counterpart in the Laurén's classification. These tumours respond well to 5-fluorouracil associated with surgery (23).

6. The role of on-slide tests: Molecular classifications using surrogate on-slide tests

The precise molecular landscape continues to be in evolution and while the most effective and reliable method for characterising the various subtypes remains the subject of debate, it is now desirable to devise a working molecular classification for GC that can be adopted widely using the current diagnostic histopathology framework. It should be noted that any GC classification is just one of a number of factors that the multidisciplinary teams/tumour boards consider when determining the best treatment for a patient. The stage of the disease, overall health of the patient and availability of different treatment options are all key elements in the decision making process (24).

Molecular stratification of GC can provide insights about the underlying biology, which may have important implications for treatment decisions. However, it is not currently possible to deliver worldwide molecular classification using the tools employed by TCGA or ACRG. Such tools are costly, not widely available, and there is a lack of capacity for the rapid turnaround time required for critically-ill patients. A fall-back position is to devise a series of on-slide tests that histopathologists can implement more widely and can be performed on formalin-fixed paraffin-embedded (FFPE) tissue.

Setia *et al* (25) provided an excellent example of such an approach. In 2016, they published a GC classification using only on-slide tests [immunohistochemical (IHC) and *in situ* hybridization (ISH)]. Initially, they evaluated 15 biomarkers using FFPE tissue from a cohort of 146 cases of GC and ultimately condensed GC into five clusters using only four on-slide tests. The authors adopted a hierarchical approach, similar to that of the molecular classification of endometrial carcinoma now ratified by the WHO (26).

Cluster 1. GC-EBV(+) (5%). This group is associated with marked lymphoid infiltrate, high PD-L1 expression and has an improved survival rate.

Cluster 2. GC-dMMR (16%). This is characterised by loss of MLH1 and PMS2 in 96% of cases, has lower rate of nodal metastasis and improved survival rate.

Cluster 3. GC-E-cadherin(-) (21%). These tumours have aberrant E-cadherin expression and contain predominately tumour of Laurén's diffuse type. This group is associated with low rate of p53 mutation compared with the other clusters. It is further divided into Cluster 3A (40%) showing complete loss of E-cadherin and Cluster 3B (60%) showing granular cytoplasmic staining of E-cadherin. Cluster 3B contains more elderly patients than 3A.

Cluster 4. GC-p53m (51%). This has aberrant p53 expression, contains predominantly tumours of Laurén's intestinal type, is associated with high Her2 expression, has more often

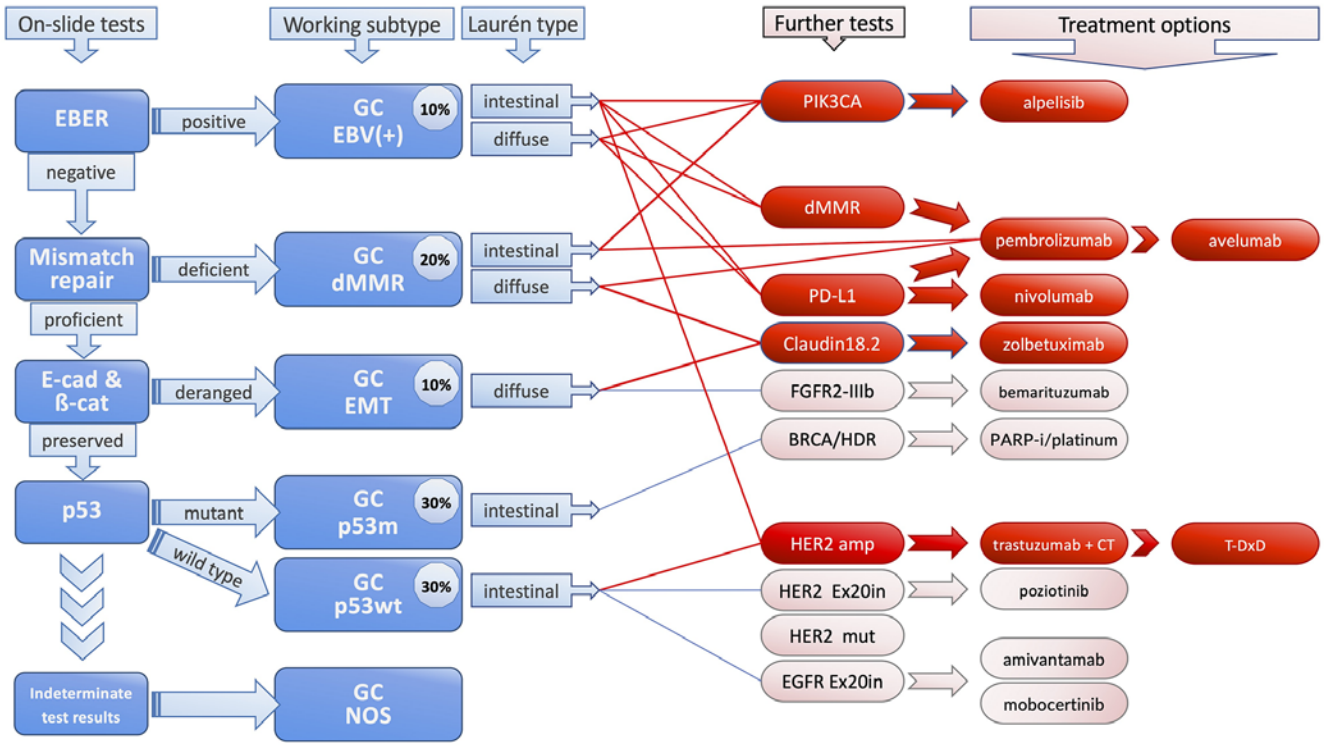

Figure 1. Proposed working classification. On the left side, the five on-slide tests required for classification are presented. The working classification comprises of 6 subtypes. The gastric cancer 'not otherwise specified' subtype is reserved for all cases with indeterminate test results. Further companion diagnostics may be selected after the Laurén's types have been considered. The therapeutic options which are more widely available at present are in red, while the others (in pink with blue lines) are mostly in pipelines. EBER, Epstein-Barr encoding region; E-cad, E-cadherin; β-cat, beta-catenin; EBV, Epstein-Barr virus; dMMR, mismatch repair deficiency; EMT, epithelial-mesenchymal transition; p53m, mutant p53; p53wt, wild-type p53; NOS, not otherwise specified; T-DxD, trastuzumab deruxtecan.

carcinoma within lymphatics and lymph node positivity. Based on IHC expression of MUC and CD10, Cluster 4 is subdivided into four subgroups.

Cluster 5. GC-p53wt (7%). This has normal p53 expression, includes tumours that lack EBV or dMMR and has no defect of E-cadherin expression. All these tumours are of the Laurén's intestinal type.

More recently, others have used the same portfolio of on-slide tests and the same subclassification. For example, Ramos *et al* (27) demonstrated that such an approach is viable in a prospective study. Importantly, they highlighted the potential difficulty in classifying tumours when expression of these four markers is heterogeneous. They raised the issue of sampling bias, thus recognising the importance of correctly interpreting mixed profiles. Ahn *et al* (28) tested a retrospective cohort of GC patients using tissue microarrays (TMAs) and showed similar correlation with prognosis. Zhao *et al* (29) used retrospective tissue in TMAs stained by IHC for mismatch repair proteins (PMS2, MLH1, MSH2 and MSH6), E-cadherin and p21 to classify CG into four subtypes, which associate with different prognoses.

7. Additional companion diagnostic biomarkers useful in GC

The adoption of a working classification would be greatly helped by providing clear links to specific treatments and recommendations on when additional tests should be performed, with a particular emphasis on biomarkers relevant for treatment selection. Some of these biomarkers are already in clinical routine use and others not yet in mainstream use for GC.

Her2. The frequency of HER2 mutation in GC has been reported to be as high as 7.7% in a previous study (30); however, a lower average of ~4.5% is more often reported (31-33). HER2 gene amplification represents a major proportion of these mutations, probably ~50%, and results in Her2 protein overexpression. In these patients, humanised anti-Her2 monoclonal antibodies, including trastuzumab, can be used successfully either to block Her-2 function, or to approach a toxic payload, including trastuzumab deruxtecan (1,34,35). Other HER2 mutations are putative resistance mechanisms to trastuzumab in Her2-positive GC. Some HER2 mutations within the active tyrosine kinase (TK)-domain, such as Ex20 insertions, are targetable by specific TK inhibitors (TKIs) (36). While a small amount of data is available on the exact incidence of HER2 activating mutations in GC (37), they are likely to represent at least 25% of total HER2 mutations (38).

EGFR. This protein is overexpressed in 27-64% of GC and can be targeted by humanised anti-EGFR monoclonal antibodies, including cetuximab or nimotuzumab (39,40). Overexpression is not always associated with gene amplification. Sensitising and resistance mutations in EGFR are known to exist in other tumour sites however no data is available on their incidence in GC.

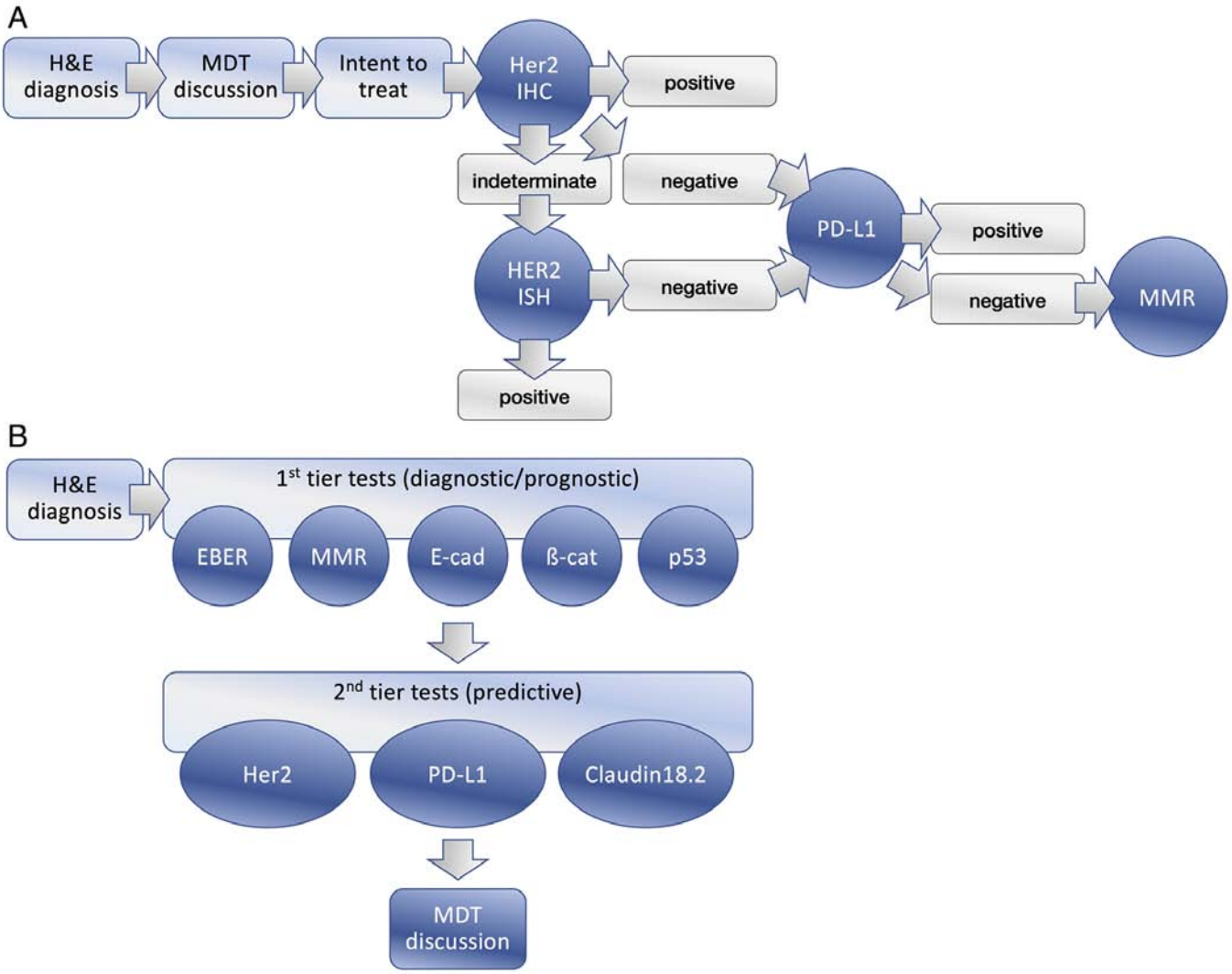

Figure 2. Laboratory workflow for on-slide tests. (A) Current mandatory laboratory workflow, slow TAT. Current step by step approach often used is demonstrated. This leads to slow TAT due to the need of interpretation of each test before the order of the next one. (B) Modified laboratory workflow, rapid TAT. After histological diagnosis of gastric cancer, all tier 1 tests are requested at the same time; tumours are classified using the hierarchical approach; second tier tests for the prediction of response to specific treatments are then reflexed accordingly. This approach on other tumour types allowed the consistent report of these datasets within two to four days TAT. TAT, turnaround time; H&E, haematoxylin and eosin; MDT, multidisciplinary team; EBER, Epstein-Barr encoding region; E-cad, E-cadherin; β-cat, beta-catenin.

PD-L1. PD-L1 selects for eligibility to immune checkpoint inhibitors (anti-PD-1, anti-PD-L1 or anti-CTLA-4) such as pembrolizumab, durvalumab, nivolumab or ipilimumab (41-43).

PIK3CA. Mutations in the catalytic domain of PIK3CA are the third most frequent mutations in GC. These mutations are associated with more aggressive behaviour and are present in 9-12% of non-hypermutated and 32% of hypermutated tumours (44). PIK3CA mutations are associated with GC-EBV(+) and GC-dMMR clusters. The most common mutation is H1047R in Ex20, which has a predilection for the GC-dMMR cluster (45). Tumour harbouring PIK3KA activating mutation can be targeted by TKIs.

KRAS. In TCGA study, KRAS mutations occur in 23% of all cases, although other studies report different penetrance, from 4 to 23% (21,46). Regardless of their incidence, there are strong correlations between KRAS mutations and the GC-dMMR cluster. At present, the only targetable KRAS mutation is G12C; this has a low prevalence, between 0.33% or 2/595 patients and 0.6% or 9/1401 patients (47-49).

ALK. The incidence of ALK rearrangement in GC is low and possibly <1% (50,51) and may be associated with dMMR. Carcinomas with ALK translocation respond well to TKIs.

MET. Amplification of MET is probably frequent in GC (52,53). Its prevalence differs in various studies, from 50% *in vitro* (52,54) to 20% *in vivo* (54-57). However, in a small cohort of 38 locally advanced GC, polysomy of Ch 7 rather than gene amplification was the reason for the increased number of MET genes per cell (58).

Claudin18.2. Part of a large family of transmembrane proteins involved in tight junctions, claudin18.2 arises from differential splicing of mRNA and is overexpressed is some GC cases, particularly those of diffuse Laurén type (59,60). It is targeted by antibody-drug conjugates, bispecific antibodies (zolbetuximab) and cell therapies such as chimeric antigen receptor T-cells (61). The mechanism of action of zolbetuximab is either via antibody-dependent cellular cytotoxicity or

complement-dependent cytotoxicity. The identification of GC with overexpression of claudin18.2 is likely to become soon mandatory (62).

8. An inclusive working classification

Recent advances in personalised treatment have provided renewed pressure to abandon traditional taxonomic classifications in favour of molecular classifications. GC has unfulfilled needs with a large proportion of patients potentially eligible for treatments that could improve outcomes significantly. It is important to define parameters for a classification of GC that enables improved access to such treatments and harmonises diagnostic categories and nomenclature. The longevity of the Laurén classification is testament to the strength inherent to the taxonomic approach and should be retained. The majority of the classifications using on-slide tests only inform some of the oncological treatments; there is a need for additional tests to select the appropriate therapy. A working classification should therefore incorporate all the currently required biomarkers and make provision for any future companion diagnostics.

In the present study, minor modifications of the nomenclature were proposed in order to reflect in an improved way current knowledge underlying these subgroups; some annotation regarding the potential therapeutic approaches was provided and possible additional companion diagnostic tests that should be considered were indicated. This proposal is summarised in Fig. 1. Through acquired knowledge from other tumour sites (e.g. endometrium), it is considered that for all cases with controversial or uninterpretable test results an indeterminate category should be added (63). The present study's classification used EBV-ISH, MMR status by IHC, E-cadherin and beta-catenin IHC and p53 IHC, which are all tests that can be delivered by most histopathology laboratories.

An important consideration for large-scale implementation is cost and impact on laboratory capacity. While a step-wise approach would have reduced the number of tests required, it would have a negative impact on TAT and would have increased the indirect costs of the laboratory to the extent that these would have exceeded any savings in reagents. It was demonstrated in other tumour types that performance of all the necessary tests up fronted results in considerable savings and allowed clinicians to have all the data within a few days (64). A first tier of on-slide biomarkers would be likely to provide prognostic data and instruct a second tier of predictive biomarkers for therapy (Fig. 2). As part of the proposed evolution of this classification, the feasibility of its implementation on a cohort of GC using FFPE have been assessed.

9. Discussion

There are a number of GC classifications based on different tests. Some of the diagnostic categories have considerable overlap, however this is not always clear, since they use different terminology. The authors consider that a first step towards widespread implementation of a working classification is the characterization of a terminology that associates the subgroups with the test results. The 'cascade' approach that has been used successfully in the WHO classification of endometrial carcinoma was adopted (26).

The modified working classification proposed here has a major focus on treatment options to help histopathologists as well as oncologists. Similar to the classification of endometrial carcinoma, there is aspiration that working molecular classification will become established in the diagnostic routine. The limited capacity for further companion diagnostic tests is well known; the working classification recognises associations between diagnostic groups and specific targeted treatments and therefore is focused on further tests regarding subgroups that are more likely to benefit. In this classification, it is relatively easy for further tests to be added as new targeted treatments become available. In Fig. 1, thin blue lines were used for treatment options that may emerge from clinical trials to exemplify how new tests can be added to the model.

Recent studies using artificial intelligence revealed that systems can be built to recognise molecular changes from H&E sections (65,66). Therefore, it is plausible that pathologists may eventually be able to recognise various molecular subtypes of GC from unique morphological features without the adjunctive help of the molecular data. This would certainly be useful for all those clinicians who do not have access to molecular tools. As demonstrated in other tumour sites, subclassification by molecular changes using surrogate on-slide markers, is a step towards this (63).

The proposed classification of the present study is a pragmatic approach to aid current GC patients. A major limitation is the relatively small number of available on-slide tests when compared with the multi-omics approach. However, whilst other classifications that use multi-omics may be more accurate in identifying relevant subgroups, their widespread implementation is currently not possible.

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Authors' contributions

SC searched the literature for similar work and articles. SC, MS, SW and CD contributed to writing the manuscript. All authors read and approved the final version of the manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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