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Review Article

Claudin-18.2 Immunohistochemical Evaluation in Gastric and Gastroesophageal Junction Adenocarcinomas to Direct Targeted Therapy: A Practical Approach

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ABSTRACT

Claudin-18.2 (CLDN18.2) expression evaluated by immunohistochemistry is a new biomarker for gastric and gastroesophageal junction adenocarcinomas that will soon have market authorization for implementation into routine clinical practice. Despite successful testing in the setting of clinical trials, no specific practical testing guidelines have been proposed. Several preanalytical and analytical variables may interfere with adequate CLDN18.2 staining interpretation; thus, this article provides practical guidance on CLDN18.2 testing and scoring in gastric and gastroesophageal junction adenocarcinomas to identify patients who may respond to targeted therapy with monoclonal antibodies directed against CLDN18.2. Based on available data, moderate to strong (2+/3+) membrane staining in ≥75% of adenocarcinoma cells is the proposed cutoff for clinical use of monoclonal antibody anti-CLDN18.2 (zolbetuximab).

Introduction

The portfolio of biomarkers used to identify patients for targeted treatment of gastric and gastroesophageal junction adenocarcinomas has been recently expanded by the introduction of claudin-18.2 (CLDN18.2) immunohistochemical evaluation in the advanced/metastatic setting for treatment with monoclonal antibody targeted to this molecule.[1-5] At present, no specific recommendations on CLDN18.2 expression assessment have been proposed, and several preanalytical and analytical variables may interfere with adequate staining interpretation. To address this issue, an international panel of pathologists (M.F., T.K., K.A.M., C.R., J.R.), with extensive experience in gastrointestinal pathology, tissue biomarker testing/reporting standardization, and CLDN18.2 immunohistochemical testing, met virtually in 3 half-day sessions between May and July 2023 to discuss the requirements for adequate CLDN18.2 immunohistochemical interpretation and reporting in gastroesophageal adenocarcinomas. A series of virtual slides obtained from 51 cases of gastroesophageal adenocarcinomas were independently reviewed by all pathologists and then jointly discussed to reach a consensus score (www.CLDN182.com).

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Both CLDN18 staining (43-14A clone; Roche Ventana) and the matched hematoxylin and eosin–stained slide were available for review. The recommendations generated from these sessions are summarized in this article and are intended as a framework for the evaluation and scoring of CLDN18.2 immunohistochemical staining in gastric and gastroesophageal junction adenocarcinomas.

Current Biomarker Testing in Advanced/Metastatic Gastric and Gastroesophageal Junction Adenocarcinomas

The relatively recent comprehensive molecular characterization of gastric and gastroesophageal junction adenocarcinomas has significantly facilitated the improvement of targeted and more effective treatments into clinical practice.[6,7] Of note, most gastroesophageal adenocarcinomas are locally advanced or metastatic at the time of diagnosis, when a curative surgical approach is no longer an option.[8] As a result, the assessment of several tissue-based biomarkers has become a mandatory step toward a personalized oncologic treatment approach in this setting.[6,9-13]

HER2 is one of the first predictive biomarkers introduced into routine practice and was the first biomarker tested in gastroesophageal adenocarcinomas.[11,14-16] Following the successful introduction of immune checkpoint inhibitors in clinical practice, immunohistochemical evaluation of programmed cell death ligand-1 (PD-L1) expression has become central in the diagnostic evaluation of patients with locally advanced or metastatic disease.[17-19] Moreover, immunohistochemical assessment of the DNA mismatch repair (MMR) protein complex is also recommended.[6,9,20] Therefore, HER2, PD-L1, and dMMR/microsatellite instability assessment of the patient's biopsy sample is now required before initiation of the first-line treatment in all patients with advanced/metastatic gastroesophageal adenocarcinomas.[6,11] Moreover, the predictive value of several other immunohistochemical and molecular biomarkers (eg, FGFR2b) is being evaluated in clinical trials and may enter routine clinical practice within the next few years, further complicating the current diagnostic testing landscape.[21,22] Most recently, CLDN18.2 testing is now ready for use[20] as it has received market authorization in some countries with Food and Drug Administration approval expected shortly within the United States.

Claudin-18.2

CLDN18.2 Is an Isoform of Claudin-18

The *CLDN18* gene, coding the claudin-18 protein, is located on chromosome 3q22.[23] The protein consists of 2 extracellular loops, 4 transmembrane domains, and a cytoplasmic domain. The first exon of *CLDN18* can be alternatively spliced, resulting in 2 different splice isoforms, CLDN18.1 and CLDN18.2, that are expressed mainly in normal lung and gastric tissues, respectively.[24,25]

CLDN18.2 is an isoform of claudin-18 and a member of a class of transmembrane proteins (ie, the claudins), which are components of tight junctions between epithelial cells.[23,24,26] In normal gastric mucosa, CLDN18.2 plays a role in maintaining barrier function, acid resistance, and cell polarity.[23,24,26] The protein is noted to be expressed in differentiated gastric cells but not in the stem cell zone.[27]

CLDN18.2 as a Novel Therapeutic Target

In 2008, Sahin et al[27] demonstrated that CLDN18.2 is a gastric lineage-specific cell surface molecule and a putative novel target for gastric adenocarcinoma using data mining and wet laboratory approaches. In normal gastric tissue, CLDN18.2 is restricted to tight junction complexes of gastric mucosal epithelial cells, limiting the access of its epitopes to intravenous antibodies. In contrast, during malignant transformation, the loss of cell polarity exposes the CLDN18.2 epitope, making it more accessible for targeting.[28]

Zolbetuximab (VYLOY; Astellas Pharma, initially termed IMAB362 or claudiximab) is a chimeric immunoglobulin G1 monoclonal antibody characterized by high specificity and affinity to CLDN18.2 and able to mediate cell death of CLDN18.2-positive cancer cells through antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity.[29] Zolbetuximab monotherapy has demonstrated a manageable safety profile, tolerability, and signs of activity in phase I clinical trials, including in patients who have been heavily pretreated. For these patients, CLDN18.2 positivity was defined as the presence of membranous tumor staining, regardless of intensity.[29,30] The following 3 phase II trials further demonstrated that zolbetuximab monotherapy or in combination with chemotherapy is well tolerated and has antitumor activity in tumors where CLDN18.2 expression was set at $\geq$50%, $\geq$70%, or $\geq$75% cutoffs.[1-3] Two recent phase III registrational randomized trials, SPOTLIGHT and GLOW, were published showing improved patient outcomes with zolbetuximab in combination with chemotherapy in the first-line setting for the treatment of CLDN18.2-positive, HER2-negative tumors where CLDN18.2 positivity was set at $\geq$75% of tumor cells.[4,5]

Based on these results, use of zolbetuximab has been proposed as a new standard of care in patients with CLDN18.2-positive, HER2-negative, locally advanced, and metastatic gastric and gastroesophageal junction adenocarcinomas. Additional trials are currently investigating possible synergies between CLDN18.2 targeting and immunotherapy.[31]

The treatment landscape for patients with CLDN18.2-positive neoplasms is rapidly evolving with the introduction of novel therapeutic options characterized by innovative mechanisms of action, such as bispecific and trispecific monoclonal antibodies,[21,24,31,32] which may have the potential advantage of improving T-cell cytotoxicity with acceptable safety and chimeric antigen receptor T-cell–based therapies.[33]

CLDN18.2 Expression in Gastroesophageal Adenocarcinomas

Diffuse, moderate to strong membranous expression of CLDN18.2 characterizes a significant proportion of gastric and gastroesophageal junction adenocarcinomas,[21,34-44] but several adenocarcinomas show complete absence of CLDN18.2 expression compared with the surrounding nonneoplastic gastric epithelium, which serves as an internal positive control.[45] Here, the normal gastric epithelium shows a strong "dark chocolate" brown intensity of membranous expression with a chicken wire distribution in the foveolar cells when 3,3'-diaminobenzidine is used as the chromogen.

Of note, precursor lesions (intestinal metaplasia and dysplasia) stain similar to the invasive adenocarcinomas but should not be included within the scoring evaluation.[46] It is essential to understand that treatment selection is based on expression within

invasive adenocarcinoma and not within areas of metaplasia/dysplasia.

The prevalence of CLDN18.2 positivity varies among studies[21,34-44] because of the use of different clones of the primary antibody, differing cutoff points for positivity, methods of stain interpretation, tumor histology/location, and sample type (ie, biopsy vs surgical resection) used for immunohistochemical evaluation. The largest series of analyzed samples demonstrated a prevalence of 38.4% (1730/4507) in gastric and gastroesophageal adenocarcinomas characterized by moderate to strong CLDN18.2 immunoreactivity in at least 75% of tumor cells.[47]

Several retrospective studies have investigated the clinicopathologic characteristics and prognostic implications of CLDN18.2-positive tumors. Some authors observed a higher prevalence of CLDN18.2 positivity in poorly cohesive carcinoma or diffuse-type carcinoma of the Laurén classification,[34,37] but this association was not consistently confirmed.

CLDN18.2 expression has been associated with tumors with a gastric phenotype based on mucin immunohistochemical profiling,[35,45,48] suggesting a possible tumor-suppressive role in the pathogenesis of tumors with an intestinal phenotype. A similar association of CLDN18.2 expression with a gastric phenotype has been reported in small bowel adenocarcinomas.[49]

In the metastatic setting, CLDN18.2 expression is highly concordant in matched primary and metastatic tumors.[34,37-39,43,50] Moreover, there is no association between CLDN18.2 expression and tumor stage.[51] It has been suggested that a higher prevalence of CLDN18.2 expression is noted among cases with peritoneal disease,[39] but this is an unconfirmed finding.

Most studies have not demonstrated a relationship between CLDN18.2 expression and prognosis, especially in the advanced/metastatic setting and in patients who received the standard first-line chemotherapy.[35,39,42,51] However, conflicting data have been published, and these findings should be prospectively validated.

From a therapeutic point of view, there are no clear differences in the prevalence of current biomarkers, including HER2, PD-L1, and MMR with claudin18.2 expression with a limited overlap between the different predictive biomarkers.[21,39,40,42,51] Wang et al[43] recently demonstrated a significantly higher prevalence of PD-L1 positivity in CLDN18.2-positive cases in a series of 451 gastric adenocarcinomas. In the same series, they found significantly higher CD4/CD8-positive T-cell fractions in CLDN18.2-positive tumors. Similar data on CD8+ T lymphocytes were published by Jia et al[40] in a series of 80 gastric adenocarcinomas, of which 60 were treated with anti-PD1/PD-L1 therapy.

In the gastric adenocarcinoma setting, a specific fusion gene involving *CLDN18*, the *CLDN18::ARHGAP*, has been described in the 15% of genomically stable tumors according to The Cancer Genome Atlas Research Network results.[52] Several authors demonstrated an association of this fusion with younger age, diffuse-type histology, metastatic disease, and worse prognosis.[53-55] Nakayama et al[54] found that all the tumors harboring this fusion showed overexpression of claudin-18.

Further confirming that only the 18.2 isoform is gastric specific, a secondary analysis of gene expression profiling data obtained on 416 gastric adenocarcinomas within the frame of The Cancer Genome Atlas demonstrated that only a 4.6% of samples were characterized by a barely detectable expression (ie, 10-100 fragments per kilobase of exon model per million reads) of claudin-18.1 isoform, although no case showed a significant level of expression for this isoform.[56]

CLDN18.2 Testing

CLDN18.2 expression is evaluated by immunohistochemistry with several clones currently available. The heterogeneity of antibodies, coupled with different staining interpretation systems, is the main cause of the different rates of CLDN18.2 expression present in the literature. Therefore, defining a standard for immunostaining and scoring protocols will be critical for generating prospectively comparable data and selection of patients eligible for treatment.

The first anti-CLDN18.2 rabbit polyclonal antibody was produced by Zymed (now Thermo Fisher Scientific) and first used in discriminating CLDN18.2 status in the MONO trial.[3] Ganymed Pharmaceuticals AG developed the CLAUDETECT 18.2 class 1 in vitro diagnostic assay based on its proprietary 43-14A clone together with BioNTech Diagnostics GmbH (formerly Theracode GmbH) and used this in vitro diagnostics in the FAST trial.[2] Ganymed (subsequently acquired by Astellas Pharma, Inc) entered into a codevelopment partnership with Ventana Medical Systems, Inc to develop an investigational automated immunohistochemical staining assay on their platform. This led to the introduction of the Ventana CLDN18 (clone 43-14A) assay for formalin-fixed, paraffin-embedded neoplastic tissue stained on the BenchMark IHC/ISH instrument.[39] This immunohistochemical assay recognizes the C terminus of claudin-18 and is not specific for the isoform 18.2.

Other clones, such as the anti-CLDN EPR19202 kit (Abcam), which was developed against a synthetic peptide within human CLDN18.2 (amino acids 1-100), are able to detect only the 18.2 isoform.[35] A comparison of the EPR19202 and 43-14A clones showed good reproducibility when detecting high CLDN18.2 expression.[38] These data can be explained by the fact that in gastric and gastroesophageal junction mucosa, CLDN18.1 expression is negligible,[27] and 43-14A immunoreactivity in this anatomical setting is reflective of CLDN18.2 expression alone. Other available CLDN18 antibodies include (1) rabbit monoclonal antibody 34H14L15 clone (Abcam); (2) mouse monoclonal 14F8 clone (CARsgen made in-house), the CLDN18 43-14A clone (Abcam), or the PathPlus claudin-18 antibody LS-B16145 clone (LifeSpan BioSciences); and (3) rabbit polyclonal antibodies (Thermo Fisher Scientific and Novus Biologicals).[33,36,44]

In a recent ring trial assessing the reproducibility and comparability of 3 CLDN18 antibodies and immunohistochemical staining platforms across a cohort of 27 global laboratories,[57,58] the analytical performance (accuracy, sensitivity, and specificity) of the Ventana CLDN18 (43-14A) assay was ≥95% and reproducible across the 27 laboratories. Moreover, the analytical performance of the 43-14A clone was equivalent to the LSBio antibody when stained on the Dako or Leica platform, although the performance was least consistent for the Novus antibody compared with 43-14A clone staining.

Looking at the complexity of the diagnostic landscape, CLDN18.2 assessment is relatively straightforward. A cell is considered positive when there is crisp membranous staining (complete, basolateral, or lateral).[27,39] Granular membrane staining, nuclear staining, and cytoplasmic immunoreactivity should not be interpreted as positive for scoring purposes.

Staining intensity is scored between 0 and 3+ (absent, 0; weak, 1+; moderate, 2+; and strong, 3+) (Fig. 1A). Also for CLDN18.2 assessment, the HER2 "magnification rule" can be easily translated into clinical practice[15]: (1) A 3+ intensity is defined as a strong brown immunoreactivity with an evident chicken wire distribution at low power (ie, ×2.5-×5 objective/×25-×50 magnification); if a higher magnification is required to confirm

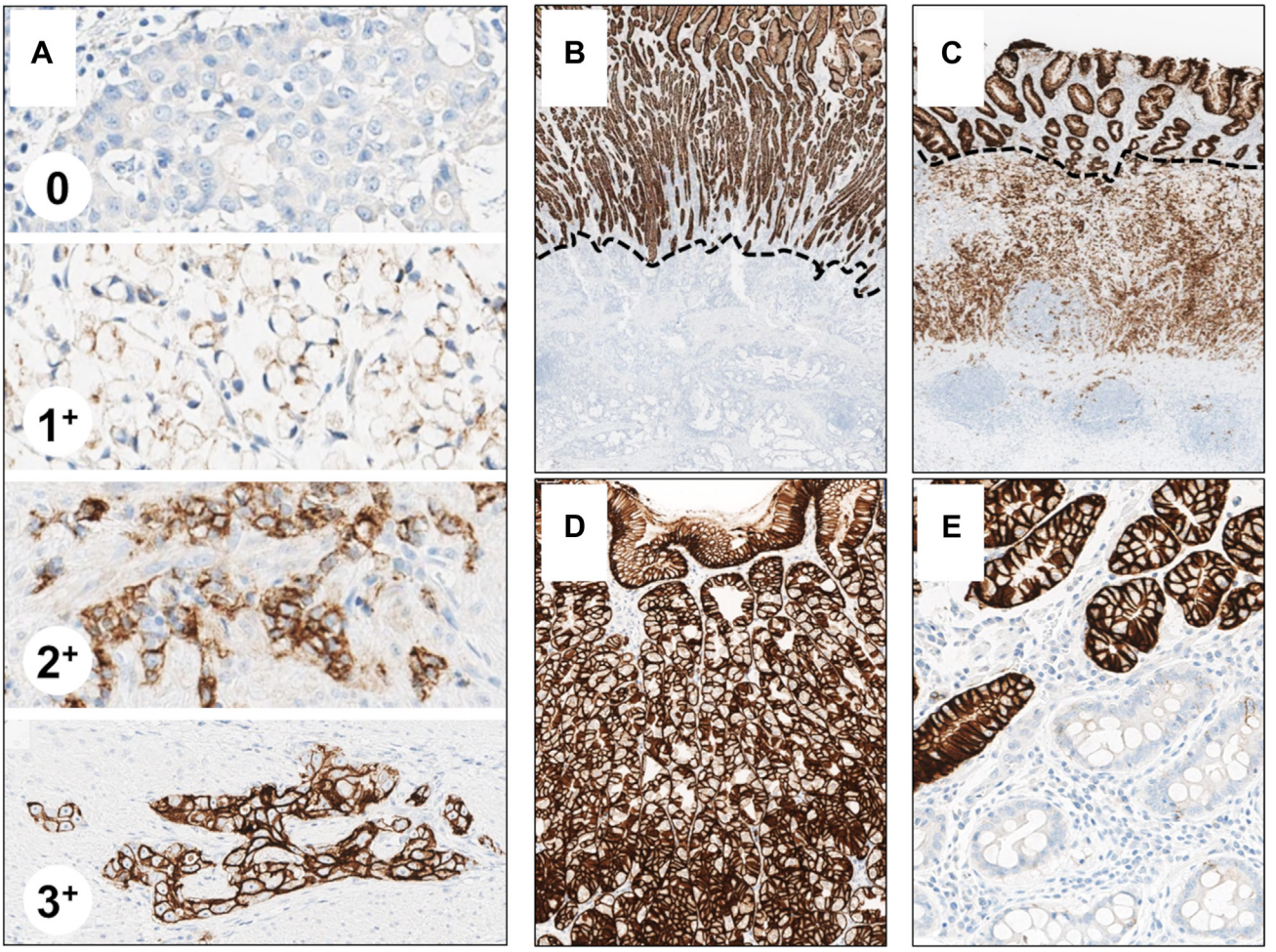

Figure 1.
Representative examples of CLDN18.2 immunohistochemical staining intensities. (A) Representative examples of 0 to 3+ CLDN18.2 staining intensity in gastric cancer cells. (B) Nonneoplastic gastric glands (above the dashed line) exhibit strong membranous immunoreactivity in contrast to a CLDN18.2-negative tumor (below the dashed line) and (C) a CLDN18.2-positive tumor (below the dashed line) gastric adenocarcinoma. (D) High magnification of normal gastric glands showing an intense cell membrane immunoreactivity with a chicken wire distribution. (E) A focus of CLDN18.2-negative intestinal metaplasia in an atrophic gastritis sample; note the positive nonmetaplastic glands (original objectives: ×5, ×10, ×20, and ×40).

membrane expression, then the evaluation is usually 1+ or 2+ for membranous staining intensity; (2) 2+ membranous staining is visible using a ×10 objective, and ×20 may be needed for confirmation of specific membrane staining; and (3) 1+ staining can be visible at ×20 but needs a ×40 objective for confirmation and should be interpreted as negative[59].

To perform an adequate evaluation of the specimen (Table), a low-magnification (×5 objective) inspection should first be performed to obtain a general impression of the staining pattern and heterogeneity of expression across the slides (Fig. 1B, C). In heterogeneous cases, the prevalence of all the 4 intensity categories (0-3+) should be recorded to more accurately evaluate the true prevalence of moderate to strong (2+/3+) immunoreactivity.

Nonneoplastic mucosa, in particular of foveolar type, is a valuable internal control to assess preanalytical issues and potential effect on staining intensity and thus serves as the maximal 3+ staining intensity in a given sample (Fig. 1D). Of note, 3+ staining of adenocarcinoma cells may be present at a slightly lower intensity in comparison with that observed in nonneoplastic foveolar mucosa.

Both the extent of staining (ie, positive tumor cell prevalence; range, 0%-100%) and H score (defined as 3 × percentage of strongly staining cells + 2 × percentage of moderately staining cells + percentage of weakly staining nuclei; it gives a range of 0-300) were used among various publications. However, based on the results of the SPOTLIGHT and GLOW trials, moderate to strong (2+/3+) positive membrane staining in ≥75% of tumor cells should be considered the standard cutoff for the interpretation as a CLDN18.2-positive tumor. Owing to different (pre)analytical factors and interpathologist variability affecting the overall CLDN18.2 expression, cases considered 2+/3+ positive in the range of 60% to 80% should be evaluated by a second pathologist.

Practical Approach to CLDN18.2 Immunohistochemical Evaluation

Sample Management and Immunohistochemical Staining

CLDN18.2 testing in clinical practice should follow standard immunohistochemical procedures on routinely processed,

Table
Essential requirements for adequate CLDN18.2 expression evaluation in gastric and gastroesophageal adenocarcinomas

Sample characteristics
• For biopsy samples from primary tumor, at least 6 tissue fragments should be evaluated; if fewer fragments are available, testing of all available tissue specimens of the tumor should be performed.
• Only invasive adenocarcinoma should be considered for scoring purposes; avoid samples consisting of only dysplastic/noninvasive lesions.
• At least 50 viable malignant cells should be evaluated, but at least 100 are preferred in effusion cytology.
• CLDN18.2 expression is highly concordant in matched primary and metastatic lesions; choose the most suitable available sample.
• In mixed-type adenocarcinomas, select the sample containing representation of the different phenotypes.
• Tissues subjected to electrocautery or with evident underfixation should not be considered for CLDN18.2 testing.
Analytical requirements
• CLDN18.2 staining should be performed on routinely processed, formalin-fixed, paraffin-embedded tissues.
• Antigen stability on unstained slides is maintained for at least 45 days. This may be extended based on validation studies in each laboratory.
• Both isoform-specific or pan–claudin-18 antibodies can be used with comparable results because of the negligible expression of the CLDN18.1 isoform in gastric tissues.
Staining evaluation
• A cell is defined as CLDN18.2 positive in the presence of crisp membranous staining (complete, basolateral, or lateral).
• Granular cytoplasmic staining is considered negative.
• Staining intensity is scored between 0 and 3+ (absent, 0; weak, 1+; moderate, 2+; and strong, 3+).
• A 3+ intensity is defined as a strong brown immunoreactivity with an evident chicken wire distribution at low magnification (×5 objective), when 3,3'-diaminobenzidine is used as a chromogen.
• CLDN18.2 staining shows a high degree of intratumoral heterogeneity.
• A low-magnification inspection of the stained slide should be completed to determine an overall impression of the staining pattern and heterogeneity of expression.
• Normal gastric glands are CLDN18.2 positive and show the maximal staining intensity in a given sample.
• False-negative staining should be considered if a sample is devoid of any staining and in cases with unstained adenocarcinomas that lack internal nonneoplastic positive controls. Repeat staining or testing of an alternative tissue block (if available) is advised.
• Precursor lesions (both intestinal metaplasia and dysplasia) may express CLDN18.2 but should not be included in the scoring.
• Normal gastric epithelium and intestinal metaplasia may be used as appropriate controls in routine practice.
• Moderate to strong (2+/3+) positive membrane staining in ≥75% of tumor cells is the proposed cutoff required for the clinical use of monoclonal antibody anti-CLDN18.2 zolbetuximab.
• Especially in poorly cohesive carcinomas, joint evaluation of CLDN18.2 staining and its matched hematoxylin and eosin–stained slide is strongly suggested.
• Cases initially considered 2+/3+ positive in the 60%-80% of cancer cells by a single pathologist should be reviewed by a second pathologist.
• Ambiguous staining should be interpreted as inadequate for CLDN18.2 scoring, and a separate sample should be obtained for retesting and scoring purposes.
Reporting of the results
• Type of specimen used for analysis (biopsy/surgical) and site of the sampling (primary/metastatic disease and if metastatic define the site).
• Sample adequacy and if inadequate state the cause.
• Antibody clone and immunohistochemical stainer used for the testing.
• Laboratory-developed test vs companion diagnostic.
• Results should be reported as the percentage of cells with moderate to strong (ie, 2+/3+) membranous immunoreactivity with a positive cutoff value of ≥75% of tumor cells.

formalin-fixed, paraffin-embedded tissues in accordance with guidance from the College of American Pathologists Pre-analytics for Precision Medicine Project Team.[60] Tissue sections should be cut at approximately 4 μm with a range from 3 to 5 μm and should be mounted onto positively charged slides. Cut slides should be desiccated and stored at room temperature (30 ± 5 °C). Antigen stability on unstained slides is maintained for at least 45 days, although environmental factors are known to affect antigen stability on cut slides, and laboratories should validate the stability within their own laboratory environment beyond 45 days. It is strongly recommended to cut sections from the tissue block immediately before testing to preserve antigenicity. Staining procedures should follow manufacturers' protocols. It is important to ensure complete removal of paraffin wax during the staining procedures as failure to do so can significantly impact staining specificity and increase background.

Positive and Negative Controls

In routine assessment of predictive biomarkers, use of optimal run controls, including positive and negative tissue/cellular samples, is strongly recommended. These controls should be prepared using similar fixation and paraffin-embedding methods to the test samples. When possible, on-slide controls with defined CLDN18.2 expression levels should be included on each sample slide.

Several positive and negative controls are commercially available for most of the current biomarker portfolio. However, it is not easy to set an adequate CLDN18.2 positive/negative control. The main difficulties for setting up adequate and reproducible standards reflect the fact that tight junctions are dysregulated in monolayer cell lines leading to inconsistent CLDN18.2 levels, whereas xenograft models exhibit some intratumor heterogeneity in CLDN18.2 expression, limiting their use as validation controls.

For practical purposes, nonneoplastic gastric tissue from a test sample can serve as an internal positive run control. Validation controls comprising tumor cores of different CLDN18.2 status are commercially available as well (eg, Discovery Life Sciences; https://dls.com/cldn182/).

Intestinal metaplasia is a suitable control for CLDN18-positive and CLDN18-negative staining elements.[46] In fact, within the same samples, there is often coexistence of strong membranous staining in normal gastric epithelial cells, weak to moderate membranous staining of epithelial cells in areas of metaplasia, and complete absence of staining in the lamina propria (lymphocytes, macrophages, smooth muscle cells, blood vessels, and peripheral nerves) (Fig. 1E).

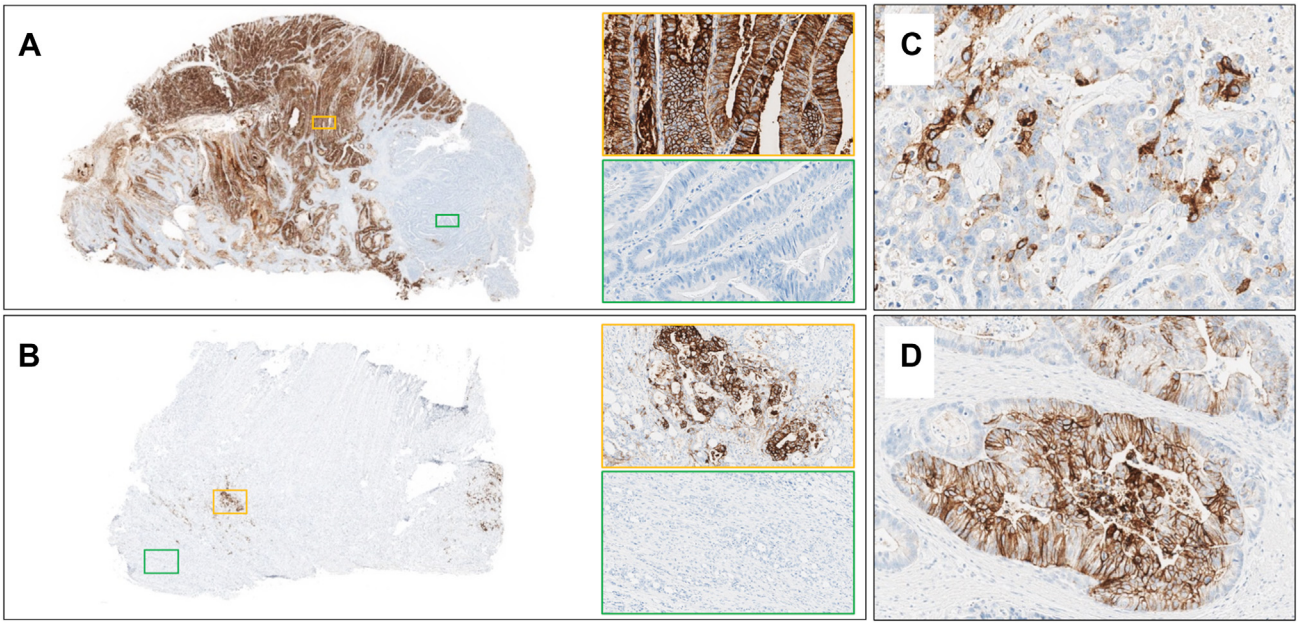

Figure 2.
CLDN18.2 heterogeneity in gastric cancer. (A, B) Two gastric adenocarcinomas characterized by evident intratumoral heterogeneity with areas showing strong intensity of CLDN18.2 expression coexisting with areas with complete absence of biomarker expression. (C, D) Two representative cases of intraglandular CLDN18.2 heterogeneity (original objectives: ×1, ×20, and ×40).

Can the Sample Type Impact CLDN18.2 Testing Results?

Both surgical specimens and biopsy samples are acceptable for CLDN18.2 testing, and the availability of specimen type may vary according to region. As previously supported for HER2 testing,[15] biopsies are usually subjected to more standardized fixation conditions and therefore are preferred to surgical samples to ensure optimal testing results.

Similar to that noted for HER2, CLDN18.2 expression frequently shows a high degree of intratumoral heterogeneity[39] (Fig. 2). Two types of heterogeneity can be considered: one is characterized by discrete areas of the tumor having absent/faint staining and a subtler form where there is coexistence of different staining intensities within the same tumor gland/small tumor area.

Intratumoral heterogeneity may affect biomarker evaluation when selecting patients who may benefit from targeted therapies, especially in primary tumor biopsy specimens.[10] Therefore, CLDN18.2 testing is best performed on an adequate number of viable endoscopic biopsy fragments (ideally 6-8). In cases of inadequate tumor sampling, all available tissue for the patient should be stained and evaluated for CLDN18.2 expression. Some institutions perform immunohistochemical predictive biomarker testing on tissue microarrays to limit the testing-related costs; however, tissue microarrays are not ideal samples for CLDN18.2 testing to inform clinical decision-making because of heterogeneous expression.

In a large retrospective single institutional study, the prevalence of significant intratumor variability in CLDN18.2 expression was observed in approximately 40% of gastric adenocarcinomas and 33% of gastroesophageal junction adenocarcinomas.[34] The same research group performed a virtual biopsy analysis by digitally scanning images of 93 surgically resected adenocarcinomas (77 gastric and 16 gastroesophageal junction adenocarcinomas).[39] Here, they simulated biopsy specimens taken during endoscopy by randomly selecting multiple (ie, 10 for each tumor) superficial tumor areas and found that sensitivity in adequately assessing CLDN18.2 expression in comparison with the results obtained in whole-slide imaging progressively increased with the number of biopsies. A small increase in sensitivity was seen after 6 biopsies, which is in line with what has been demonstrated with HER2 testing in gastroesophageal adenocarcinomas.[10,14,15] These data confirm that at least 6 biopsies from a primary tumor should be available to adequately evaluate CLDN18.2 expression.

Is it Better to Test Primary or Metastatic Disease?

Several studies investigated the correlation of CLDN18.2 expression between primary tumors and synchronous/matched lymph nodes or distant metastases.[34,37-39,43,50] A high concordance rate (>80%) was described in most available series; therefore, both tissue samples can be considered adequate and equivalent for CLDN18.2 testing. Moreover, Dai et al[50] demonstrated a high concordance rate (83.7%) between peritoneal cytologic effusion specimens and matched surgical pathology biopsies and/or resection specimens.

There are currently no data available on whether specific metastatic sites (eg, the peritoneum and liver) can influence CLDN18.2 expression or if CLDN18.2 expression changes during tumor progression (ie, synchronous vs metachronous metastases) or after neoadjuvant/adjuvant therapies. The assessment of CLDN18.2 expression in (often limited) biopsy material from metastatic sites can be challenging, especially in peritoneal samples, in which there may be few malignant cells (Fig. 3). A minimum of 50 viable neoplastic cells should be required for an adequate evaluation of CLDN18.2 expression. However, similar to the other predictive biomarker testing (eg, PD-L1),[17] 100 viable malignant cells is a better cutoff point for quantitative evaluation.

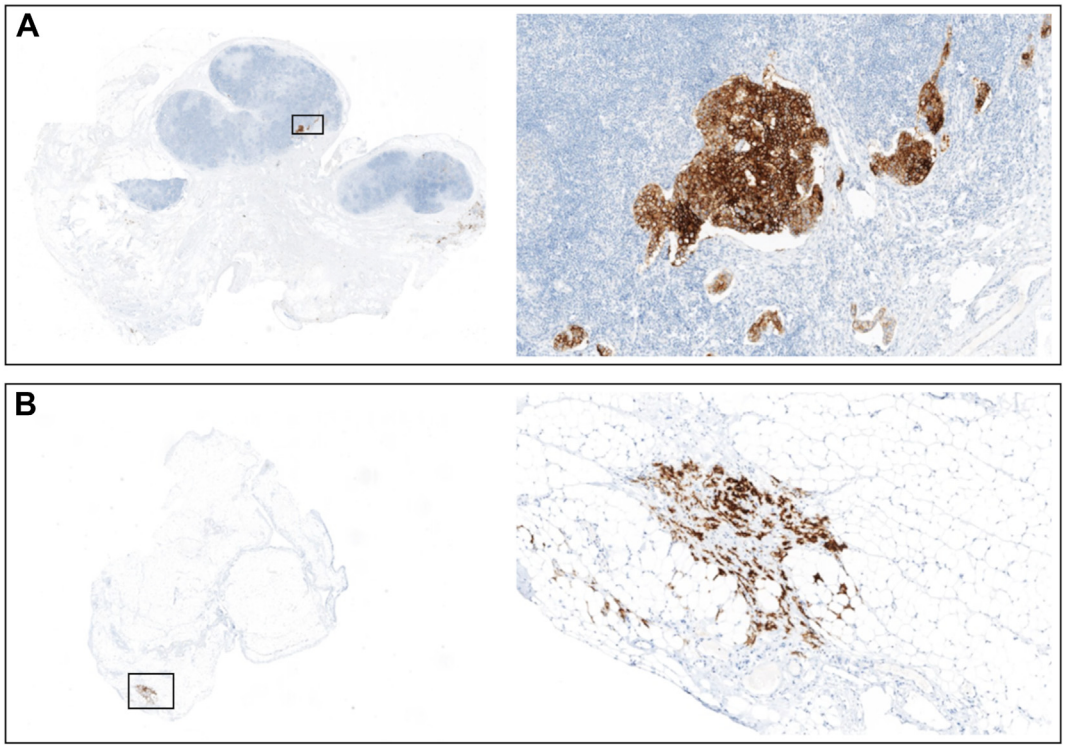

Figure 3.
CLDN18.2-positive metastatic adenocarcinoma. Three cases of CLDN18.2-positive cases of metastases with limited amount of tumor content but with 3+ CLDN18.2 expression. (A) A lymph node micrometastasis; the evaluation of the matched hematoxylin and eosin–stained slide is necessary to adequately evaluate tumor extent. (B) A peritoneal metastasis with a limited number of cancer cells; at least 50 viable tumor cells (preferably 100 cells) are suggested for CLDN18.2 scoring (original objectives: ×1 and ×20).

Tumor Histology and CLDN18.2 Expression

Some reports demonstrated a higher prevalence of CLDN18.2 expression in adenocarcinomas showing poorly cohesive histology/diffuse subtype by the Laurén classification.[34,37] This association was more evident for the signet ring cell subtype. A comprehensive analysis of the 4507 tested cases among the different clinical trials demonstrated a statistically significant association between CLDN18.2 expression and tumor type ($P = .0002$); patients with poorly cohesive carcinoma/diffuse subtype tumors (48.3%; 553/1145) had the highest prevalence of CLDN18.2 positivity compared with those with intestinal (38.8%; 308/794) and mixed-type tumors (42.9%; 134/312).[47]

Care should be taken when evaluating poorly cohesive adenocarcinomas as negative tumor cells may be overlooked and mistaken for inflammatory or stromal cells on immunostained slides. To overcome the challenge in assessing the true extent of tumor (and therefore the percentage of CLN18.2-positive cells), a matched hematoxylin and eosin–stained slide from the same tumor block should always be evaluated. When possible, evaluation by or with a gastrointestinal pathologist is also advised.[61]

In cases with mixed histology (intestinal and poorly cohesive), the most representative tumor sample/block exhibiting morphologic tumor heterogeneity should be selected for CLDN18.2 testing. In fact, CLDN18.2 expression may be different in the 2 neoplastic constituents, but the report should represent a comprehensive evaluation of both components.

Adenosquamous carcinoma is a rare variant of esophageal and gastroesophageal junction carcinomas. Only rare cases have been tested for CLDN18.2 expression, and the squamous component always lacked expression.

Gastric well-differentiated neuroendocrine tumors and linear and nodular enterochromaffin-like hyperplasia frequently show moderate to strong CLDN18.2 expression.[46] Therefore, mixed neuroendocrine/nonneuroendocrine neoplasms and gastric adenocarcinomas arising from a background of autoimmune gastritis should be carefully evaluated. Only the adenocarcinomatous component is scored and reported. In these cases, evaluation of matched chromogranin A and hematoxylin and eosin–stained slides can assist in identifying areas appropriate for scoring.

Potential Pitfalls in CLDN18.2 Assessment

- *Cytoplasmic or nuclear staining.* A central dogma of CLDN18.2 assessment is that only tumor cells with perceptible and convincing linear membranous staining should be considered positive. Cytoplasmic or (more rarely) nuclear staining is not scored. In cases with high CLDN18.2 expression, membrane expression may be obscured by strong cytoplasmic staining, but usually, a chicken wire pattern can still be observed (Fig. 4).
- Signet ring cells may be misinterpreted because of nonspecific staining in the marginated cytoplasm similar to that noted for HER2.[62] It is not always easy to discriminate whether granular positivity in the cytoplasm is associated with clear linear membrane expression. Only definitive linear membrane expression should be considered. In cases with ambiguous staining, cautious interpretation is recommended. In the

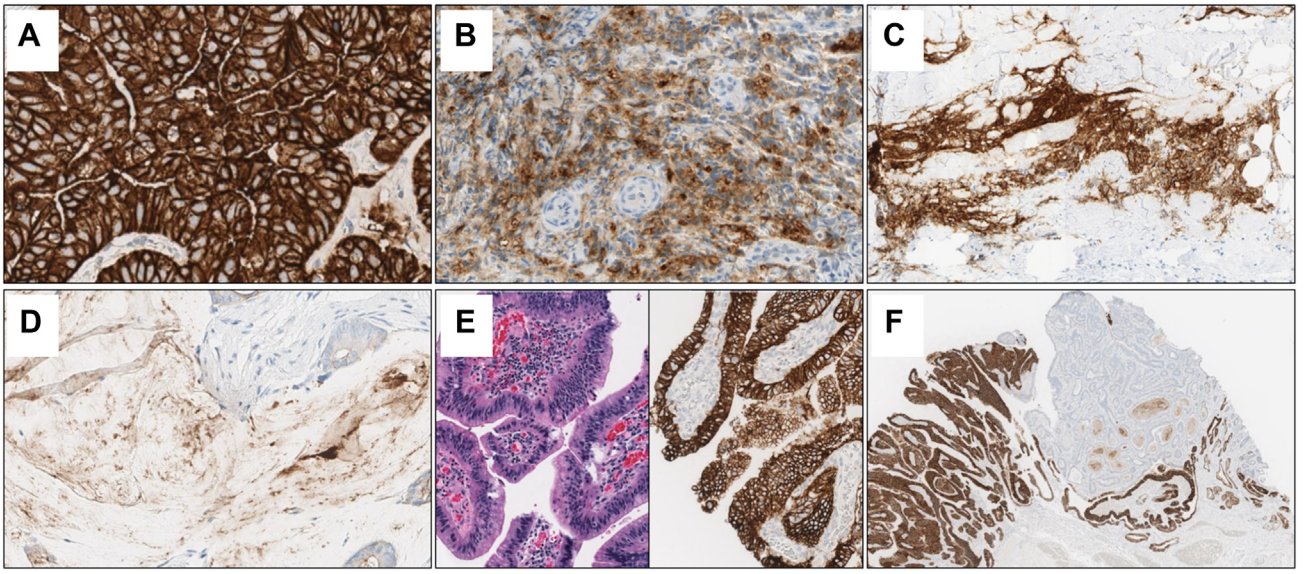

Figure 4.
Potential pitfalls in CLDN18.2 immunohistochemical evaluation. (A) Extensive cytoplasmic staining in a strongly CLDN18.2-positive adenocarcinoma makes evaluation of the membrane staining difficult. (B) A gastric cancer characterized by granular cytoplasmic immunostaining. (C) Thermal/cautery effect in a CLDN18.2-positive adenocarcinoma. (D) Faint to moderate expression in acellular mucin/mucus. (E) Low-grade dysplasia with strong and diffuse CLDN18.2 expression. (F) Low-grade and high-grade dysplasia exhibiting a heterogeneous pattern of staining; all epithelial elements in this image are dysplastic (original objectives: ×5, ×10, ×20, and ×40).

presence of significant artifacts (preanalytic, thermal effect, etc) associated with cytoplasmic staining, the case should be interpreted as "inadequate for CLDN18.2 evaluation," avoiding the possibility of false-negative or false-positive results. In these cases, an alternative sample from the lesion should be obtained for scoring purposes. If only a part of the tumor is acceptable for scoring, while the remainder is characterized by evident artifact, scoring should only be performed in stained regions void of artifact.

- *Dysplastic lesions.* Gastroesophageal dysplastic lesions (both low and high grades) show CLDN18.2 expression similar to that observed in invasive adenocarcinoma.[46] However, there are no data available on the concordance of CLDN18.2 expression in dysplastic lesions and their matched invasive counterparts. Several reports demonstrated that dysplasia expression can be discordant with expression in the associated adenocarcinoma[9] and should not be considered in the assessment of CLDN18.2 expression.
- *Aberrant positivity.* Rare cases showing aberrant faint cytoplasmic positivity in inflammatory cells or other non-neoplastic cells have been observed and should not be scored. Most of these cases were characterized by obvious preanalytical factors, and CLDN18.2 staining should be repeated on another sample when available. Aberrant moderate to strong positivity in acellular mucin from cases of mucinous adenocarcinomas or within necrotic cellular debris can be observed and should prompt careful evaluation of the specimen.
- *Preanalytical artifacts.* The preanalytical phase is a common and important source of error in biomarker testing. Tissue subjected to electrocautery (especially from mucosal/submucosal dissections) appears histologically as torn and coagulated. In these samples, antigen preservation may be impaired, and areas with thermal artifact should be excluded from CLDN18.2 evaluation. Underfixation may also be detrimental for antigen maintenance and may cause false-negative staining with edge effect and nonspecific cytoplasmic staining.

Ideal CLDN18.2 Pathology Report

Standardization of pathology reports and biomarker interpretation are critical for personalized oncologic treatments. In fact, the pathology reports should present results in a clear and concise manner to guide oncologists in the selection of the best treatment options. An ideal CLDN18.2 expression report should contain the following information:

- type of specimen used for analysis (biopsy/surgical) and site of sampling (primary/metastatic disease and if metastatic define the site)
- tissue fixation time
- sample adequacy, and if inadequate, the causes of inadequacy should be explicitly stated (eg, lack of tumor cells, electrocautery/fixation artifacts, and <6 tested biopsies)
- antibody clone and immunohistochemical stainer used for the testing
- companion diagnostic or laboratory-developed test
- test results as the percentage of membranous moderate/strong (2+/3+) positive cells
- test results as positive/negative according to the ≥75% of membranous positive (2+/3+) adenocarcinoma cell cutoff and
- additional analytical and clinical interpretative comments (eg, qualification of the laboratory and bibliographical references), if needed.

Conclusions

CLDN18.2 expression tested by immunohistochemistry will soon enter daily clinical practice for the selection of patients with locally advanced and metastatic gastric and gastroesophageal junction adenocarcinomas who may benefit from anti-CLDN18.2 therapeutic approaches. We have attempted to highlight the most important aspects of CLDN18.2 staining and interpretation

along with potential pitfalls, as observed in real-world practice and as assessed in 51 cases of gastroesophageal adenocarcinomas independently reviewed by a group of pathologists working in collaboration with the developers of zolbetuximab to optimize patient selection.

Accurate evaluation of predictive biomarkers is the foundation of current precision oncology paradigm, and pathologists play a central role in therapeutic decision-making. This is even more important in the gastroesophageal cancer setting, in which most immunohistochemical/molecular testing is performed on limited biopsy material.

Author Contributions

All authors equally contributed in the writing and conceptualization of the manuscript. All authors have read and agreed to the published version of the manuscript.

Data Availability

All data/information are available upon request to the corresponding author.

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Declaration of Competing Interest

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Ethics Approval and Consent to Participate

All information regarding human material was managed using anonymous numerical codes, and all samples were handled in compliance with the Declaration of Helsinki.

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