Original Article

Claudin 18 immunohistochemistry in cholangiocarcinoma

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Background: Monoclonal antibodies against claudin (CLDN) 18.2 (a component of tight junctions) in gastric epithelial cells are an emerging therapeutic option for patients with advanced gastric and esophageal adenocarcinoma. Phase 2 and 3 trials have shown clinical efficacy in patients whose tumors show high expression of CLDN18 by immunohistochemistry, and the US Food and Drug Administration has recently approved a drug for patients with advanced gastric and gastroesophageal adenocarcinoma and high CLDN18 expression. Adenocarcinoma of the bile ducts, a.k.a. cholangiocarcinoma (CCA), may share morphologic and immunophenotypic qualities with gastric adenocarcinoma, and are lethal tumors with limited therapeutic options. The purpose of this study was to determine if primary tumors of the bile ducts show expression of CLDN18 with the use of a monoclonal antibody to CLDN18, and if so, if the extent of expression is similar to that seen in the gastric and esophageal tumors of patients who responded to anti-CLDN18.2 therapeutics.

Methods: Tissue microarrays containing 41 intrahepatic cholangiocarcinomas, 36 hilar cholangiocarcinomas, and 28 distal bile duct cholangiocarcinomas were stained with a monoclonal antibody which detects CLDN18 (Ventana 43-14A). The percentage of tumor cells staining and intensity was recorded for each case with tumors with 75% of cells showing moderate to strong intensity being considered high expressers.

Results: High expression was seen in 14.63% of intrahepatic, 8.3% of hilar, and 17.8% of distal bile duct cholangiocarcinomas. Overall, 13.33% of CCAs expressed CLDN18 to an extent which would qualify for treatment in the gastric and esophageal trials.

Conclusions: Given the poor prognosis and current lack of therapeutic options, trials of anti-CLDN18.2 inhibitors could be considered in patients with CCA and high expression of CLDN18 by immunohistochemistry.

Keywords: Claudin 18.2 (CLDN18.2); cholangiocarcinoma (CCA); anti-CLDN18.2 monoclonal antibody

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Introduction

Claudins (CLDNs) are widely expressed tight junction proteins that play a critical role in maintaining the integrity of epithelial barriers. Different cell types express different CLDNs in varying amounts. CLDN18 has 2 main isoforms—CLDN18.1 which is normally expressed in lung, and CLDN18.2 which is normally expressed in gastric mucosa (1,2). Studies have shown that CLDN18.2 is aberrantly expressed in a few types of solid tumors, including pancreatic adenocarcinoma and cholangiocarcinoma (CCA) (1,3,4). Several fairly large studies have shown no positive or negative association between CLDN18 expression by immunohistochemistry and survival (in the absence of targeted therapy) (5-8). CLDN18.2 is normally embedded within the tight junction, but disturbed polarity in malignancy increases its exposure (1,9). This makes CLDN18.2 an attractive target for cancer therapy, particularly for those tumors that are difficult to treat using traditional approaches. In this regard, CLDN18 immunohistochemical positivity does not appear to be correlated to other "theranostic" tests which are commonly performed on gastric and esophageal adenocarcinoma such as immunohistochemistry for human epidermal growth factor receptor 2 (HER2/neu) and programmed death ligand-1 (PD-L1) (5,7).

Following promising preclinical studies, several clinical trials have been completed to evaluate the safety and efficacy of CLDN18.2-targeted therapies in patients with advanced gastric, gastroesophageal, and esophageal adenocarcinoma. The FAST trial which consisted of patients with advanced gastric, gastroesophageal, and gastroesophageal junction tumors receiving an anti-CLDN18.2 monoclonal antibody (zolbetuximab) plus chemotherapy *vs.* chemotherapy alone showed marked improvement of progression free survival and overall survival in patients with 2+ or 3+ immunohistochemical reactivity in 70% of tumor cells. This trial had originally considered 40% positive tumor cells (with 2+ or 3+ intensity) to be positive, however patients with between 40–69% of cells staining did not demonstrate clinical benefit (9). The SPOTLIGHT trial, a large phase 3 trial comparing zolbetuximab plus typical chemotherapeutics (FOLFOX) to FOLFOX alone in patients with advanced gastric or gastroesophageal adenocarcinoma recently demonstrated positive results for the zolbetuximab arm (10). Notably, SPOTLIGHT used a cut-off of 75% of tumor cells showing moderate to strong expression. The GLOW trial ran concurrently and tested the efficacy of zolbetuximab combined with a different chemotherapy regimen (CAPOX) than that used in SPOTLIGHT. It showed very similar positive results for the zolbetuximab arm (11). These trials provoked considerable interest among clinical oncologists and oncologic surgeons, and zolbetuximab was recently approved by the US Food and Drug Administration for patients with advanced gastric or gastroesophageal adenocarcinoma. We now test all invasive gastric, gastroesophageal, and esophageal adenocarcinoma for CLDN18 at Columbia University Irving Medical Center.

CCA is a highly aggressive malignancy of the biliary tract epithelium. It is the second most common primary liver cancer, with a rising incidence and mortality rate (12). CCA is classified and staged according to its anatomical epicenter as intrahepatic cholangiocarcinoma (ICCA), hilar cholangiocarcinoma (HCCA), and distal bile duct cholangiocarcinoma (DCCA) (13). Despite advances in diagnostic and therapeutic modalities, the prognosis of CCA remains poor, mainly due to the limited efficacy of current treatments. CLDN18 is not present in

Highlight box

Key findings

- High expression of monoclonal antibody to claudin (CLDN) 18 was seen in 14.63% of intrahepatic, 8.3% of hilar, and 17.8% of distal bile duct cholangiocarcinomas. Overall, 13.3% of cholangiocarcinomas (CCAs) expressed CLDN18 to an extent which would qualify for treatment in the gastric and esophageal trials.

What is known and what is new?

- Anti-CLDN18.2 monoclonal antibody (zolbetuximab) was recently approved by the US Food and Drug Administration for patients with advanced gastric or gastroesophageal adenocarcinoma. CCA is a highly aggressive malignancy arising from the biliary tract epithelium. It is the second most common primary liver cancer, with a rising incidence and mortality rate.
- Despite advances in diagnostic and therapeutic modalities, the prognosis of CCA remains poor, mainly due to the limited efficacy of current treatments. This study is designed to determine what proportion of well-characterized CCA express moderate or strong CLDN18 expression in 75% or more of tumor cells (if any).

What is the implication, and what should change now?

- If a proportion of CCA are identified to express moderate or strong CLDN18 expression in 75% or more of tumor cells, that would provide a rationale to consider anti-CLND18.2 monoclonal antibodies in these patients with otherwise poor prognoses and limited therapeutic options.

Table 1 Selected studies of CLDN18 in gastrointestinal tumors

Study	Population	Antibody	Definition of positive	Result
Arnold *et al.*	381 gastric/GEJ adenocarcinoma	Ventana 43-14A	Immunoreactivity score >8 (of 12)	17.1%
Coati *et al.*	523 gastric/GEJ adenocarcinomas	Clone 34H14L15, Invitrogen	H-score (0–300) with positivity achieved at 51	29.4%
Sahin *et al.* (FAST Trial)	686 advanced gastric/GEJ/ esophageal adenocarcinomas	CLAUDETECT18.2, Ganymed	40% 2 or 3+	49% but >70% of positive patients had >70% 2+ or 3+
Shinozaki *et al.*	200 biliary neoplasms	Polyclonal antibody; Zymed	1%	43% of ICCA, 90% DCCA
Shitara *et al.* (SPOTLIGHT Trial)	2,735 gastric/GEJ adenocarcinomas	Ventana (43-14A)	75% 2 or 3+	42%
Pellino *et al.*	350 gastric/GEJ adenocarcinomas	Ventana (43-14A)	75% 2 or 3+	33.4%
Kubota *et al.*	408 gastric/GEJ adenocarcinomas	Ventana (43-14A)	75% 2 or 3+	24%
Present study	105 CCA	Ventana (43-14A)	75% 2+ or 3+	13.3%

CCA, cholangiocarcinoma; DCCA, distal bile duct cholangiocarcinoma; GEJ, gastroesophageal junction; ICCA, intrahepatic cholangiocarcinoma.

normal biliary epithelia, however studies have shown it is upregulated in biliary neoplasia. The largest study using immunohistochemistry to detect CLDN18 in biliary neoplasms used a polyclonal antibody and set a cut-off for positivity at 1% of tumor cells. Within these parameters, the proportion of positive cases ranged from 43% of ICCA to 90% of DCCA. CLDN18 positivity by immunohistochemistry was associated with negative tumor prognostic factors and worse overall survival (4). However, given that the potential use of therapeutics drives the current interest in CLDN18, and that contemporary trials require more robust tumor reactivity, the present application of the 2011 study is limited. The previous studies which are most germane to this project are summarized in *Table 1*.

This study aims to determine what proportion of well-characterized CCA express moderate or strong CLDN18 expression in 75% or more of tumor cells (if any). If so, that would provide a rationale to consider anti-CLND18.2 monoclonal antibodies in these patients with otherwise poor prognoses and limited therapeutic options.

Methods

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the institutional review board (IRB) at Columbia University Irving Medical Center (No. AAAN2452). Informed consent was obtained via our standard surgical consent form which contains a provision that authorizes using the patient's tissue for research purposes.

Tissue microarrays were constructed with 41 ICCA, 36 HCCA, and 28 DCCA cases collected between 2000 and 2016. The arrays consist of two 2 mm cores of tumor tissue, and one 2 mm core of benign tissue from the same specimen. Basic patient demographics were characterized.

Immunohistochemistry

Immunohistochemical staining was performed with the anti-CLDN18 monoclonal antibody from Ventana (43-14A). The assay was performed on the Ventana Benchmark automatic stainer according to the manufacturer's recommendations; briefly 64 minutes of heat retrieval using Ventana "CC1" buffer at 95 degrees, 20 minutes antibody incubation, and detection with Ventana Ultraview DAB detection system. Results were recorded with average intensity and percentage of positive cells with 75% or more staining with an intensity of 2+ or 3+ being considered positive/high-expressers. Only membranous staining was considered positive. All cases were reviewed by one pathologist with experience in predictive immunohistochemistry (S.M.L.). Difficult/borderline cases were reviewed with another experienced pathologist (H.R.).

If both pathologists agreed on positivity *vs.* negativity, then the average of their scores was used. If there was positive/negative discrepancy, a third experienced pathologist (H.M.K.) served as a "tiebreaker".

Molecular

The Cerner (CoPath) laboratory information system at NewYork-Presbyterian/Columbia University Irving Medical Center (New York, NY, USA) was retrospectively searched for molecular findings (including cytogenetics) in these 105 cases. Molecular and/or cytogenetics results were available for 34 of 105 cases.

Microsatellite instability

Multiplexed polymerase chain reaction (PCR) amplification of DNA extracted from tumor and paired normal tissues was performed using the Promega MSI Analysis System version 1.1, with fluorescently labeled primers specific for five mononucleotide markers and two pentanucleotide markers. PCR amplification was followed by capillary electrophoresis.

Next-generation sequencing: Columbia Combined Cancer Panel

Targeted exonic and intronic sequence was obtained from DNA purified from tumors (with or without paired normal DNA) using Custom Agilent Sureselect capture and Illumina HiSeq2500 sequencing. Samples had average coverage of at least 500-fold, and at least 50-fold coverage of greater than 98% of coding sequences in the regions of interest. These sequences were evaluated for single nucleotide variants, and small insertions and deletions.

Next-generation sequencing: targeted panels

Sequence data was obtained via probe extension and ligation, followed by multiplexed PCR with adapters for Illumina sequencing, and sequencing on the Illumina MiSeq platform with reversible fluorescent terminators. The Genes tested are listed in Table S1.

Sanger sequencing

Targeted PCR amplification of *KRAS* exon 2 was performed using flanking intronic primers. The PCR products were then treated with exonuclease plus shrimp alkaline phosphatase, dideoxyterminator sequencing with BigDye Terminator, and capillary electrophoresis.

Cytogenetics

Slides were prepared with fixed cells from cytology brushings of the bile duct. Slides were aged at room temperature. Following this, the slides were immersed in 2× saline sodium citrate (SSC) for 30 min at 37 ℃ followed by digestion in the pepsin working solution (10 µL pepsin in 50 mL 0.01 N HCl) at 37 ℃ for 10 min. Immediately after digestion, the slides were placed in a phosphate buffer solution (PBS) with MgCl2 for 5 min at room temperature, followed by 10 min at room temperature 1% formaldehyde solution. The slides were washed in PBS for 20 mins and then dehydrated using an ethanol series (70%, 85%, 100%) for 2 min each at RT. Working solution of CEP3 DNA labeled with Spectrum Orange dUTP (Abbott Molecular/Vysis Products, Illinois, USA), CEP7 DNA labeled in FITC Green dUTP (Abbott Molecular/Vysis Products) and CEP17 DNA labeled with Spectrum Aqua dUTP (Abbott Molecular/Vysis Products) was made by mixing 1 µL of each concentrated Centromere probe with 7 µL of LSI/WCP® hybridization buffer (Abbott Laboratories, Illinois, USA). Separately, a working solution of CDKN2A (p16) DNA labeled with Spectrum Orange dUTP (Abbott Molecular/Vysis Products) and CEP9 DNA labeled with FITC Green dUTP (Abbott Molecular/Vysis products) was made by mixing 1 µL of concentrated probe with 7 µL of LSI/WCP® hybridization buffer (Abbott Laboratories). Each working solution of the probe sets was applied separately to two different target areas. The slides were coverslipped, co-denatured with ThermoBrite™ at 75 ℃ for 5 min, and hybridized overnight in a 37 ℃ humidified oven. Following hybridization, slides were soaked in RT 2×SSC/0.1% NP-40 to remove coverslips, placed in 2×SSC/0.1% NP-40 at 74 ℃ for 2 min, and placed into RT 2×SSC/0.1% NP-40 for 2 min. The slides were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Newark, CA, USA), coverslipped, and analyzed by two technologists using standard fluorescence microscopy methods.

Data analysis

Microsoft® Excel® for Microsoft 365 MSO (Version 2501 Build 18429.20158 Click-to-Run) was used for all statistical calculations.

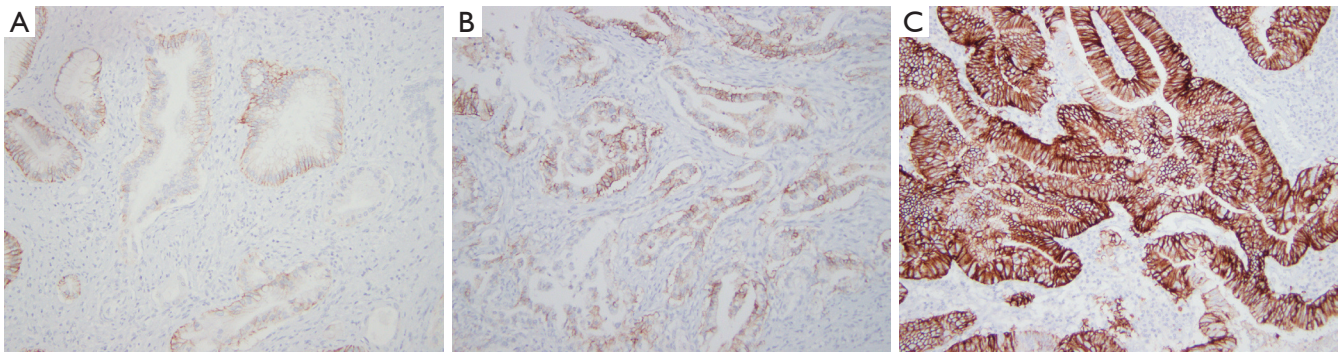

Figure 1 Representative images of CLD18 expression in cholangiocarcinoma by immunohistochemistry. Representative low 1+ (A), moderate 2+ (B), and strong 3+ (C) expression of Ventana (43-14A) (200×).

Results

The median age of the patients was 64 years and 50.5% were women. Six of 41 (14.6%) ICCA had at least 75% of tumor cells staining with moderate to strong intensity. Three of 36 (8.3%) HCCA and 5 of 28 (17.8%) DCCA showed similar positivity. Overall, 14 of 105 (13.3%) of CCA showed moderate to strong expression of CLDN18 in 75% or more of tumor cells when tested with the Ventana monoclonal antibody (Clone 43-14A). Only three cases showed marked heterogeneity between plugs. These three cases were each ultimately negative as the combined positivity was <75% (e.g., 80% in one plug and 0% in another). Of the 14 positive cases, 8 showed moderate differentiation, 3 were poorly differentiated, and 3 were well-differentiated. No morphologic features were present in the CLDN18 positive cases compared to the negative cases.

In addition to the 14 positive cases (defined as 2+ or 3+ intensity in 75% or more of tumor cells), another 25 cases showed low expression. These low-expression cases ranged from 1+ intensity in 5% of tumor cells to 2+ positivity in 70% of tumor cells (*Figure 1* and *Table 2*). Only 2 cases require a tiebreaker.

As standard clinical protocols for evaluating CCA at our institution have varied over time, we have variable amounts of molecular and cytogenetic data. No clear associations are noted (Table S1).

Discussion

CCA is a disease with high mortality (and morbidity) and limited therapeutic options. In this study approximately 13% of a well-characterized cohort of CCA shows high expression of CLDN18, which raises the possibility of administering anti-CLDN18 monoclonal antibodies as a therapeutic intervention in such cases. It does not appear that anatomical epicenter correlates with high CLDN18 expression. Similarly, differentiation does not appear to correlate to its expression. Overall, the immunohistochemical assay was quite easy to interpret. We did not encounter aberrant staining patterns (such as nuclear reactivity). Most cases were positive or negative with only 14 (13.3%) being "difficult". Kizler *et al.* reported similar positivity (13.1%) in CCA with the same antibody (VENTANA CLDN18). Isidro *et al.* have reported 24.1% of ICCA and 56% of extrahepatic cholangiocarcinoma to be positive for CLDN18, although, they have used the sigma (HPH-018446 rabbit antiserum) antibodies (14,15).

Clinical trials of anti-CLDN18.2 monoclonal antibodies have shown benefit in gastroesophageal adenocarcinoma patients with high CLDN18 expression using immunoreactivity cutoffs of 70% with 2 or 3+ positivity and 75% with 2 or 3+ positivity, and zolbetuximab has now received FDA approval in these patients. Given that we are unaware that CCA patients have ever been tried on anti-CLDN18.2 therapy, we cannot say whether the gastric/gastroesophageal junction (GEJ) cutoff is appropriate, only that it seems rational given the literature. As a practical matter, we suggest simply reporting the percentage of tumor cells with membranous staining and the intensity. In our experience, CLDN18 1+, 2+, and 3+ are like the intensity levels in HER2/neu immunohistochemical evaluation. A question that often comes up when evaluating stains of this sort is whether the whole slide should be considered, or a "hotspot", and indeed there is some heterogeneity to this assay (though not extensively so). In our opinion, it would

Table 2 Immunohistochemical results

Immunohistochemical staining with Claudin antibody	Negative	Positive (>75% cells staining)		Percentage of positive cases	Tumor differentiation (n)		
		2+	3+		Well	Moderate	Poor
Intrahepatic (n=41)	35	1	5	14.6	1	3	2
Hilar (n=36)	33	3	0	8.3	1	1	1
Distal (n=28)	23	0	5	17.8	1	4	0
Total	91	4	10	13.3	3	8	3

be inappropriate to use a hotspot approach. The existing trial literature does not describe the use of a hotspot, and both major trials (FAST and SPOTLIGHT) required high expression to elicit clinical benefit, with FAST showing no benefit to patients with <70% reactivity. Therefore, hotspot analysis could lead to treatment for patients who are unlikely to benefit and should be avoided.

CLDN18 has two isoforms (CLDN18.1 and CLDN18.2). We are currently only aware of trials involving CLDN18.2 targeting the gastrointestinal tract. The Ventana antibody is purported to react with both isoforms, so it is not impossible that we were detecting CLDN18.1, which would not be meaningful for clinical oncology. However, we consider this unlikely for several reasons. First, biliary cancers share significant overlap with gastric and esophageal adenocarcinoma concerning morphology and typical immunohistochemical profiles (16). A very recent study looked at CLDN18 expression in non-ampullary small intestinal adenocarcinoma and expression was strongly correlated to a gastric immunophenotype (expression of cytokeratin 7 and MUC5AC) (17). Lung adenocarcinoma is usually fairly distinct from upper gastrointestinal tract and biliary cancers, so logically that the CLDN18 expression we found was the CLDN18 isoform associated with gastrointestinal cancers, not pulmonary cancer. Secondly, although the Ventana assay purports to react with both isoforms, normal lung tissue was negative in our testing, whereas normal stomach was reliably positive. So, in our experience, the assay is more sensitive for CLDN18.2 than CLDN18.1. The FAST trial used the "CLAUDETECT18.2" assay, which is done manually and does not scale to clinical practice (9). The SPOTLIGHT trial, which is likely an important piece of any submission to regulatory agencies, used the same Ventana clone as we used for this study, as have many other studies (see *Table 1*) (10). The study compared sensitivity between two commercially available assays (Abcam EPR19202 and the Ventana 43-14A) and found 43-14A more sensitive (6). The lower sensitivity of the EPR19202 assay was thought to explain a study which showed lower immunoreactivity for CLDN18 (8). Therefore, we believe the Ventana assay, which is readily commercially available and compatible with autostainers, is the best assay to use.

Our study has limitations. For example, we evaluated HER2/neu, mismatch repair (MMR) proteins and PD-L1 immunohistochemistry, but antigenicity was poor on some TMAs when PMS2 was tested. Therefore, negative HER2/neu and PD-L1 results could not be confidently accepted. As such it is possible that we underestimated CLDN18 positivity rates. However, our results are roughly similar to the gastric/GEJ data, and in our opinion are sufficiently positive to justify prospective study.

Conclusions

In summary, given the excitement regarding the results of clinical trials of anti-CLDN18.2 monoclonal antibodies in patients with upper gastrointestinal tract adenocarcinoma and high immunohistochemical expression of CLDN18, we investigated whether the morphologically and immunophenotypically similar adenocarcinomas of the biliary tree showed similar patterns of expression. Regardless of the tumor's anatomical epicenter, CCA expresses CLDN18 strongly at rates roughly similar (though somewhat lower) to those observed in patients with upper gastrointestinal tract adenocarcinoma who responded well to anti-CLDN18.2 therapy. Given their poor prognosis, it is reasonable to consider whether patients with CCA and high expression of CLDN18 may benefit from CLDN18.2 inhibition.

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Footnote

Data Sharing Statement: Available at https://jgo.amegroups.com/article/view/10.21037/jgo-2024-925/dss

Peer Review File: Available at https://jgo.amegroups.com/article/view/10.21037/jgo-2024-925/prf

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jgo.amegroups.com/article/view/10.21037/jgo-2024-925/coif). S.M.L. has received honoraria for serving as a panelist for Astellas Pharmaceuticals. R.M. has received payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing, or educational events from HMP Global for the lecture Great Debates & Updates in Gastrointestinal Malignancies and from MDOutlook for an educational event on immunotherapy in Upper GI cancers; received consulting fees from Puretech Health and has participated on a Data Safety Monitoring Board or Advisory Board of IDEAYA Biosciences and Nimbus Therapeutics; has received Drug Support from Verastem and Vivace Therapeutics and Research funding for institution from Repare Therapeutics and Nimbus Therapeutics. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the institutional review board (IRB) at Columbia University Irving Medical Center (No. AAAN2452). Informed consent was obtained via our standard surgical consent form which contains a provision that authorizes using the patient's tissue for research purposes.

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