Analysis of the Expression of Cathepsin S in Gastric Adenocarcinoma and in *Helicobacter Pylori* Infection

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Abstract

Background: recent experimental studies have shown a potential link between cathepsin S (CTTS) and gastric cancer progression. Herein, we aimed to evaluate the expression of CTTS in gastric adenocarcinoma.

Methods: Cross-sectional study that included two groups, gastric adenocarcinoma (n=42) and gastritis (n=50). The gastritis group was then subdivided into H. pylori positive (n=25) x negative (n=25). Gastric tissue samples were analyzed in order to determine the CTTS expression through immunohistochemistry.

Results: In patients with gastritis, the age ranged from 18 to 78 years. Among them, 34% were male, and 66% were female. In patients with gastric cancer, the age ranged from 37 to 85 years. Among them, 50% were male, and 50% were female. When comparing the expression of CTTS between the two groups, only 16% of the gastritis samples had an expression higher than 25%. On the other hand, among patients with gastric adenocarcinoma, 19% had expression between 25-50%, 14.3% between 51-75%, and 26.2% had expressions higher than 75% ($p < 0.001$). CTTS expression was significantly higher in patients with positive test for H. pylori: 87.5% x 38.5% ($p<0.001$). There was no statistically significant association between the positivity of CTTS and the clinical-pathological variables, including tumor staging, histological type, angiolymphatic invasion, recurrence, current status and death.

Conclusion: CTTS has a higher expression in samples of gastric adenocarcinoma. Patients with gastritis by *H. pylori* also show a higher expression of CTTS compared with patients with negative results for this bacterium.

Introduction

Cathepsins are enzymes that comprise a family of 15 lysosomal proteases widely distributed in intracellular and extracellular spaces, among which five have been implicated repeatedly in the progression of solid cancers (cathepsin B, H, K, L, and S) [1, 2].

Cathepsins plays roles in a wide range of body activities based on their hydrolysis effect. In digestive cancers, the expression of cathepsin is positively regulated by tumor-promoting factors, such as C-myc, K-ras, AGR2, MAPK, p38, and the Hedgehog (Hh) signaling pathways. Activated cathepsins hydrolyze growth factors, such as EGF, VEGF and TGFβ, promoting the proliferation of cancer cells, and they appear to play a role, although still uncertain, in the regulation of apoptosis [3, 4]. In addition, analyses of expression of cathepsins in tumor microenvironments have sparked discussion about the role of these molecules in the response to anti-cancer therapy and in the phenomenon of therapeutic resistance [5].

Recently, studies have pointed to a supposed relation between gastric cancer and cathepsin expression, specifically cathepsin S (CTTS). Data are however still incipient, suggesting a therapeutic, prognostic, and diagnostic potential of this enzyme in the evolution of this disease [6]. In vitro studies have shown that increased CTTS expression is related to increased tumor invasion and metastasis, and that its inhibition

is capable of preventing tumor cell invasion and migration in gastric cancer [7, 8]. Parallel to this, studies have also shown that the serum expression of CTTS may be a great ally in the early diagnosis of gastric cancer, even presenting a sensitivity superior to the usual tumor markers, such as CEA, CA 19.9, and CA 72.4 [8].

The present study aims to evaluate the expression of CTTS in gastric tissue samples of patients with gastric adenocarcinoma and compare it with the expression in gastric tissue samples of patients without cancer, only with gastritis. In addition, this study seeks to evaluate the impacts of H. pylori infection on CTTS expression in gastric tissue samples without cancer.

Methods

Study design

This is a cross-sectional study carried out at Hospital das Clínicas, Federal University of Pernambuco, Recife, Brazil, aiming to evaluate the expression of CTTS in gastric tissue samples of patients diagnosed with gastric adenocarcinoma (n = 42) and of patients diagnosed only with gastritis (n = 50). The group of patients with gastritis was subdivided into another two subgroups: one with a positive result for *H. pylori* (n = 25) and one with a negative result for *H. pylori* (n = 25). The primary result was to compare CTTS expression assessed by immunohistochemistry in gastric tissue samples from patients with adenocarcinoma and patients with gastritis (with and without *H. pylori*).

All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional research committee and the 1964 Helsinki declaration and its later amendments, or comparable ethical standards. This research's protocol was approved by the Ethics Committee of the Hospital das Clínicas da Universidade Federal de Pernambuco (HC/UFPE-EBSERH) under the protocol CAAE no. 38000620.9.0000.8807. Informed consent was obtained from all participants in the study.

Selection of patients

We included patients undergoing surgical treatment of gastric adenocarcinoma with curative intent in our center from 2017 to 2019. We excluded patients at stage IV and those undergoing neoadjuvant chemotherapy. After each surgical procedure for the treatment of gastric adenocarcinoma, we always selected the first patient to present a confirmed histopathological result for gastritis in the Pathology sector of our institution, aiming to form the control group with the lowest possible risk of selection bias. This group included patients who underwent esophagogastroduodenoscopy (EGD) with biopsy of lesions, confirming that it was gastritis. The search for *H. pylori* was performed in all patients using the urease test and confirmed by histopathology with Giemsa stain. We excluded patients previously submitted to gastroplasty and those with reports of previous treatment for *H. pylori*.

Immunohistochemistry

We performed immunohistochemical staining (IHC) to study the expression of CTTS in 42 samples of human gastric cancer tissue (Fig. 1) and in 50 samples of gastric tissue with gastritis (Fig. 2). We made 3-μm sections in series for immunohistochemical analysis and placed them on Superfrost Plus glass slides. We performed immunostaining using the Ventana BenchMark ULTRA System automated staining system using rabbit polyclonal antibody directed against CTTS (Clone No. A13482; ABclonal, Massachusetts, United States). We used a 1:100 dilution and incubated it for 30 min to 37 ºC. We used the DAB IHC Detection Kit as the chromogen substrate. All specimens were counterstained with hematoxylin. We interpreted immunohistochemical reactions using a standard optical microscope and analyzed them according to the specific pattern of the investigated antibody. We assessed marking intensity using the following grading:

Grading	Marking
0	no detectable coloring
1	weak coloring = light yellow
2	moderate coloring = brown-yellow
3	strong coloring = brown

We graded the percentage of stained tumor cells as follows:

Grading	Stained cells
0	no positive tumor cells
1	1–25% of positive tumor cells
2	26–50% of positive tumor cells
3	51–75% of positive tumor cells
4	> 75% of positive tumor cells

We calculated the staining index score as the product of the percentage of positive tumor cells and the intensity of staining. We defined CTTS expression according to the color index:

Expression	Coloring index
-	0: negative
+	1–4: weakly positive
++	5–8: positive
+++	9–12: strongly positive

For analysis purposes, the CTTS expression intensity was categorically assessed: high expression or low expression. We defined a high expression as a color index score > 4, while low expression was a score ≤ 4. An index = 0 corresponds to a missing expression.

Statistical analysis

For statistical analysis, we used the software STATA/SE 12.0 and Excel 2010. Mean values and standard deviations were calculated to describe data population. We considered a 95% confidence for all tests and a p-value < 0.05 as significant in all tests. The results are presented in tables containing respective absolute and relative frequencies. We verified the existence of associations using the Chi-square test and Fisher's exact test for categorical variables. The statistical power of this sample was 72.7% according to the presence or absence of CTTS in the gastric cancer groups compared to benign stomach lesions.

Results

From ninety-two (92) patients studied, 50 patients had gastritis and 42 patients had gastric adenocarcinoma. In patients with gastritis, the age ranged from 18 to 78 years. Among them, 35 patients (70%) were under 50 years old, 17 (34%) were male, and 33 (66%) were female. In patients with gastric cancer, the age ranged from 37 to 85 years. Among them, 34 patients (81%) were over 50 years old, 21 (50%) were male, and 21 (50%) were female.

Regarding the tumor topography, 23 patients had tumors in the antrum (54.8%) and 19 in the gastric body (45.2%). Subtotal gastrectomy was performed in 20 patients (47.6%), and total gastrectomy in 22 patients (52.4%). As for pathological staging, ten patients were at stage IA (23.8%), ten at stage IB (23.8%), 19 at stage IIIB (45.3%), and three at stage IIIC (7.1%). Regarding histological type, 18 were classified as intestinal (42.9%), 18 as diffuse (42.9%), and six as mixed tumors (14.2%). We detected lymphatic vascular embolization of neoplastic cells in 23 cases (54.8.7%), and absent in 19 cases (45.2%). Recurrence occurred in 23.8% of cases. The situation of these patients at the end of the study was alive without disease (71.4%), alive with disease (7.1%), death from other causes (4.8%), death from cancer (16.7%). Table 1 shows the frequencies of clinical variables, staging, therapy, and follow-up of 42 patients with CG in this study.

Table 1
Characterization of the group of patients with gastric cancer.

	Gastric cancer group	
Variable	n	%
Topography		
Antrum	23	54.8
Body	19	45.2
Type of surgery		
Subtotal gastrectomy	20	47.6
Total gastrectomy	22	52.4
Pathological stage		
IA	10	23.8
IB	10	23.8
IIIB	19	45.3
IIIC	03	7.1
Primary tumor		
T1	10	23.8
T2	10	23.8
T3	19	45.3
T4	03	7.1
Lymph nodes		
N0	20	47.6
N3	22	52.4
Histological type		
Intestinal	18	42.9
Diffuse	18	42.9
Mixed	06	14.2
Histological grade		
Well differentiated	03	7.1

	Gastric cancer group	
Moderate	08	19.0
Poorly differentiated	31	73.9
Angiolymphatic invasion		
Positive	23	54.8
Negative	19	45.2
Recurrence		
No	32	76.2
Yes	10	23.8
Current status		
Alive without disease	30	71.4
Alive with disease	03	7.1
Death without cancer	02	4.8
Death with cancer	07	16.7

When comparing the expression of CTTS between the two groups, in gastritis samples 38% did not express CTTS, 46% had low expression (1–25%), and only 16% had an expression higher than 25%. On the other hand, among patients with gastric adenocarcinoma, 19% had expression between 25–50%, 14.3% between 51–75%, and 26.2% had expressions higher than 75%, with statistically significant results ($p < 0.001$). Analyses involving the CTTS staining index in IHC and the intensity of expression also showed a statistical significance, being higher in the group of patients with gastric adenocarcinoma (Table 2).

Table 2
Comparison between gastric cancer x gastritis groups

	Group				
	Gastric cancer		Gastritis		
Variable	**n**	**%**	**n**	**%**	**p-value***
Age					
Under 50	8	19.0	35	70.0	**< 0.001**
Over 50	34	81.0	15	30.0	
Gender					
Male	21	50.0	17	34.0	0.121
Female	21	50.0	33	66.0	
H. pylori					
Positive	8	19.0	24	48.0	**0.004**
Negative	34	81.0	26	52.0	
Percentage of CTTS stained cells					
No positive cells	07	16.7	19	38.0	**< 0.001**
1–25%	10	23.8	23	46.0	
25–50%	08	19.0	04	8.0	
51–75%	06	14.3	02	4.0	
> 75%	11	26.2	02	4.0	
CTTS coloring index					
Negative (0)	7	16.7	19	38.0	**0.002**
Weakly positive (1–4)	14	33.3	24	48.0	
Positive (5–8)	09	21.4	03	6.0	
Strongly positive (9–12)	12	28.6	04	8.0	
Intensity of expression					
Absent – 0	7	16.7	19	38.0	**0.001**
Low < 4	14	33.3	24	48.0	

(*) Chi Square Test.

Group				
High ≥ 4	21	50.0	07	14.0
(*) Chi Square Test.				

In the evaluation of CTTS expression in the group of patients with gastritis, CTTS expression was significantly higher in patients with positive test for *H. pylori*: 87.5% x 38.5% (p < 0.001) (Table 3).

Table 3
Expression of CTTS in the group of patients with gastritis.

CTTS coloring score					
	Positive		**Negative**		
Variable	**n**	**%**	**n**	**%**	**p-value***
Age					
Under 50	22	62.9	13	37.1	0.849
Over 50	09	60.0	06	40.0	
Gender					
Male	11	64.7	06	35.3	0.777
Female	20	60.6	13	39.4	
H. pylori					
Positive	21	87.5	03	12.5	**< 0.001**
Negative	10	38.5	16	61.5	
(*) Chi Square Test.					

In the evaluation of CTTS expression in the group of patients with gastric adenocarcinoma, there was no statistically significant association between the positivity of the expression and the clinical-pathological variables presented in Table 4.

Table 4
Expression of CTTS in the group of patients with gastric adenocarcinoma.

	CTTS				
	Positive		Negative		
Variable	**n**	**%**	**n**	**%**	**p-value***
Age					
Under 50	7	87.5	01	12.5	1.000
Over 50	28	82.4	06	17.6	
Gender					
Male	17	81.0	04	19.0	1.000
Female	18	85.7	03	14.3	
H. pylori					
Positive	27	79.4	7	20.6	0.312
Negative	08	100.0	0	0.0	
Topography					
Antrum	18	78.3	05	21.7	0.428
Body	17	89.5	02	10.5	
Type of surgery					
Subtotal gastrectomy	15	75.0	05	25.0	0.229
Total gastrectomy	20	90.9	02	9.1	
Staging					
IA	08	80.0	02	20.0	0.490
IB	07	70.0	03	30.0	
IIIB	17	89.5	02	10.5	
IIIC	03	100.0	0.0	0.0	
Primary tumor					
T1	08	80.0	02	20.0	0.490
T2	07	70.0	03	30.0	

(*) Fisher's exact test

	CTTS				
T3	17	89.5	02	10.5	
T4	03	100.0	0.0	0.0	
Lymph nodes					
N0	15	75.0	05	25.0	0.229
N3	20	90.0	02	9.1	
Histological type					
Intestinal	14	77.8	04	22.2	0.852
Diffuse	16	88.9	02	11.1	
Mixed	05	83.3	01	16.7	
Histological grade					
Well differentiated	03	100.0	0	0.0	0.177
Moderate	05	62.5	03	37.5	
Poorly differentiated	27	87.1	04	12.9	
Angiolymphatic invasion					
Positive	20	87.0	03	13.0	0.682
Negative	15	78.9	04	21.1	
Recurrence					
No	26	81.3	06	18.8	1.000
Yes	09	90.0	01	10.0	
Current status					
Alive without disease	24	80.0	06	20.0	0.475
Alive with disease	02	66.7	01	33.3	
Death without cancer	02	100.0	0	0.0	
Death with cancer	07	100.0	0	0.0	
Death					
Yes	09	100.0	0	0.0	0.314
No	26	78.8	07	21.2	
(*) Fisher's exact test					

Discussion

The cathepsins that have already shown an increased expression in the presence of gastric cancer are B, E, K, L, S, X, and Z. To date, there are only a few studies that sought to assess the relation between CTTS and gastric cancer [7, 8]. This enzyme appears to play an important role in the tumor invasion process through the degradation of the extracellular matrix, modulation of the immune response, and regulation of several cell signaling pathways, including the activation of tyrosine kinase receptors, especially c-Met, matrix metalloproteinases, IL-11, CXCL16, and Integrin alpha-6-beta-4 [2, 7, 9]. Still, specifically for gastric adenocarcinoma, CTTS appears to have an activating effect on the MKN7 and MKN45 cancer cell lines [7].

Liu et al. [8] evaluated the serum dosage of CTTS in patients with gastric cancer by comparing the results with healthy patients and with benign gastric lesions. They observed that the serum CTTS values of patients with gastric cancer were significantly higher than those of non-tumor gastric tissue controls ($P < 0.001$). Still in this study, the authors investigated the diagnostic power of CTTS in their sample of 496 patients, finding sensitivity and specificity values of 60.7% and 90%, respectively. Additionally, in this study, there was a significant decrease in serum CTTS levels after surgical resection of the tumor, suggesting an intimate relation between this enzyme and the tumor microenvironment. In our study, we found similar results, with CTTS expression significantly higher in the group of patients with gastric adenocarcinoma compared to those of controls. The results of these studies suggest that CTTS may be a potential biomarker for the diagnosis of gastric cancer.

Yang et al. [7] studied the expression of cathepsins through a proteomic analysis of cultures of normal cells and gastric cancer. We observed a higher protein expression and a positive regulation of cathepsin S in gastric cancer cell secretome. There were no statistically significant differences in CTSS expression between the intestinal, diffuse, and mixed subtypes.

Researchers have shown a correlation between CTTS and disease characteristics, such as tumor size, lymph node invasion, distant metastases, and overall survival, noting that higher CTTS expressions were related to more advanced TNM stages and worse survival rates [8]. In the present study, there was no statistically significant association between CTTS expression and tumor staging or survival rates. A possible explanation for such a difference between the studies is the number of patients included, which was noticeably lower in our analysis.

Infection of the gastric mucosa by *H. pylori* is known to be an important risk factor for the development of gastric adenocarcinoma. However, the exact mechanisms of activation of carcinogenesis are not yet fully elucidated [10]. One of the possible mechanisms pointed out in this process is the pro-inflammatory response orchestrated by Th17 cells in the infected gastric mucosa [11, 12]. Previous studies have shown an association between infection by *H. pylori* and increased levels of expression of cathepsins D and X. However, there are no studies determining the behavior of CTTS in the presence of an infection by *H. pylori* [13, 14]. In the present study, we evaluated the expression of CTTS in samples of gastric mucosa infected by *H. pylori*. We observed that 87.5% of the samples in the gastritis group with *H. pylori* showed

positive expression for CTTS, contrasting with only 12.5% of the gastritis group without *H. pylori*. These results reinforce the hypothesis that CTTS is involved in the process of carcinogenesis of gastric adenocarcinoma, since it also has a higher expression.

This study has some limitations that deserve attention. First is the sample size, which, as a result of the single-center character of this study, was limited, with a sample power of 72.7%. As the sample was non-probabilistic, and selected by convenience, we did not calculate the sample size since we included in the analysis all patients operated on during the study period. Another limitation that is worth mentioning is in relation to the observational and cross-sectional nature of this study. A longitudinal analysis could provide more accurate information about the relationship between CTTS expression and patient survival. However, for our primary result, the methodology applied was adequate.

In contrast to the limitations discussed above, the present study reports important data that provide robustness and authenticity to the analysis. It is one of the few studies to study the expression of CTTS in samples of gastric adenocarcinoma in humans and the first to attest a possible relationship between the expression of this enzyme and infection by *H. pylori*, an important risk factor for the development of gastric adenocarcinoma.

Conclusion

Considering the results of the present study, the authors conclude that CTTS has a higher expression in samples of gastric adenocarcinoma compared to samples of non-tumor tissue. In addition, as a secondary finding, we report that patients with gastritis by *H. pylori* also show a higher expression of CTTS compared with patients with gastritis with negative results for this bacterium. These results reinforce the discussion about the role of CTTS in the evolution of gastric cancer. However, further studies are needed aiming to define the relation of this enzyme in the process of gastric adenocarcinoma carcinogenesis.

Declarations

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Conflicts of interest

All authors have declared no conflicting interests.

Disclosure:

Authors have nothing to disclose.

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Figures

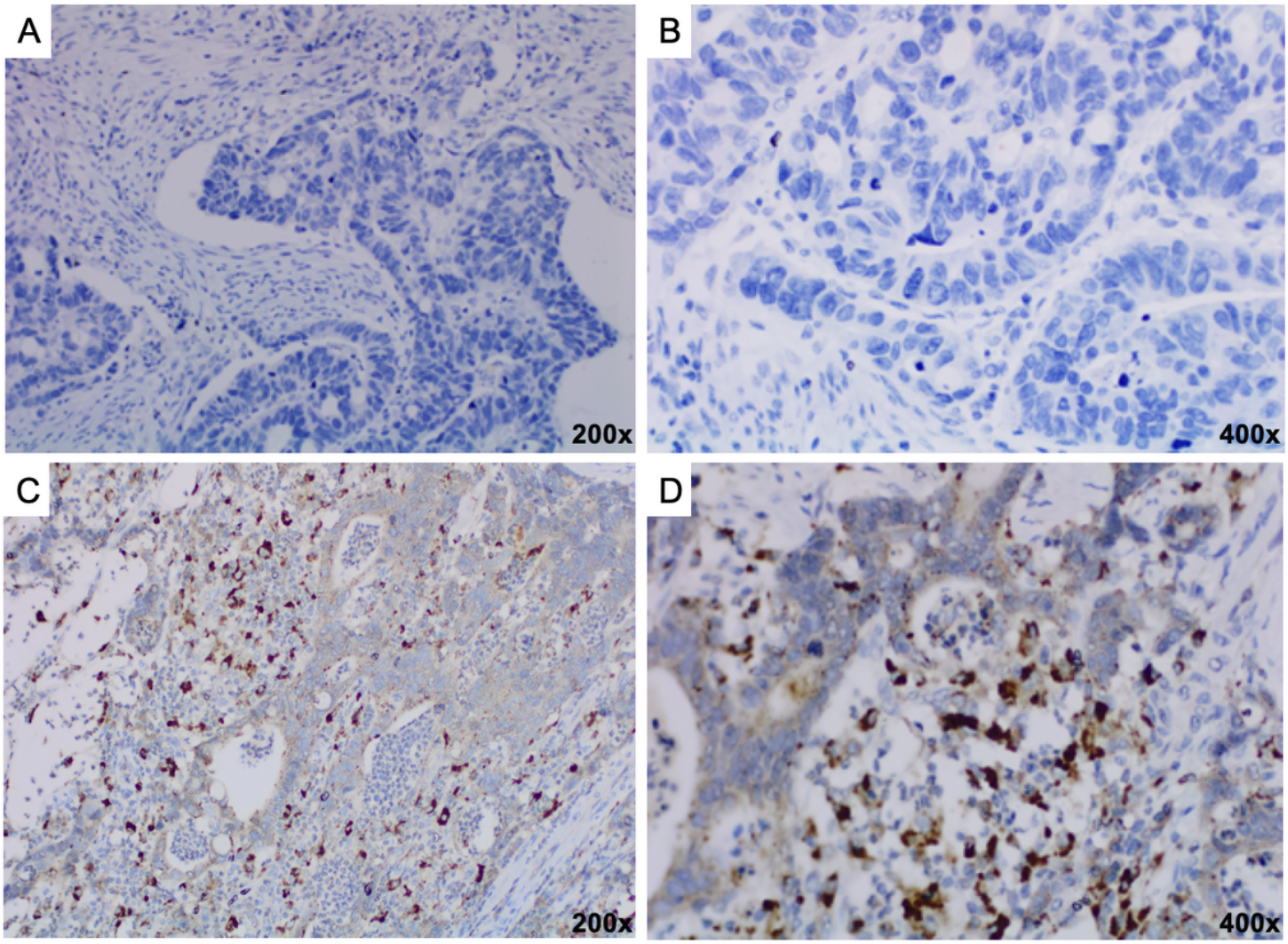

Figure 1

(A-B): IHC staining showing negative expression of CTTS in gastric adenocarcinoma; (C-D): IHC staining showing positive expression of CTTS in gastric adenocarcinoma.

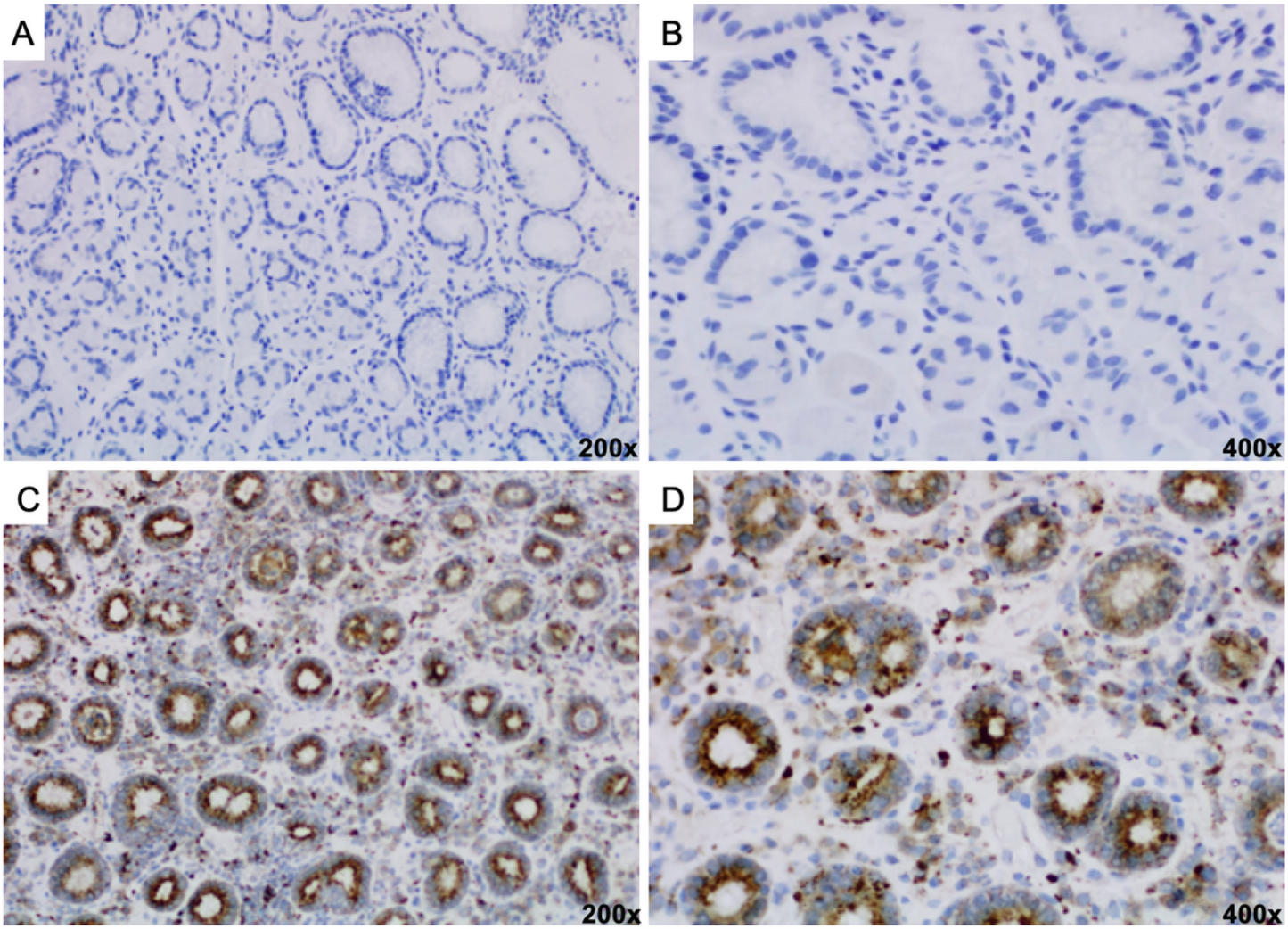

Figure 2

(A-B): IHC staining showing negative expression of CTTS in a gastric tissue sample with gastritis; (C-D): IHC staining showing positive expression of CTTS in a gastric tissue sample with gastritis.