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Antral atrophy, intestinal metaplasia, and pre-neoplastic markers in Mexican children with *Helicobacter pylori*-positive and negative gastritis

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Abstract

Chronic inflammation and infection are major risk factors for gastric carcinogenesis in adults. As chronic gastritis is common in Mexican children, diagnosis of *Helicobacter pylori* and other causes of gastritis are critical for the identification of children who would benefit from closer surveillance. Antral biopsies from 82 Mexican children (mean age 8.3±4.8y) with chronic gastritis (36 *H. pylori* +, 46 *H. pylori* -) were examined for gastritis activity, atrophy, intestinal metaplasia, and immunohistochemical expression of gastric carcinogenesis biomarkers CDX2, ephrin type-B receptor 4, matrix metalloproteinase 3 (MMP3), macrophage migration inhibitory factor (MIF), p53, β-catenin, and E-cadherin. Atrophy was diagnosed in 7/82 (9%) and intestinal metaplasia in 5/82 (6%) by routine histology, while 6 (7%) additional children (3 *H. pylori* +) exhibited aberrant CDX2 expression without intestinal metaplasia. Significant positive correlations were seen between EphB4, MMP3, and MIF ($p<0.0001$). Atrophy and follicular pathology were more frequent in *H. pylori* + biopsies ($p<0.0001$), while intestinal metaplasia and CDX2 expression showed no significant correlation with *H. pylori* status. Antral biopsies demonstrating atrophy,

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intestinal metaplasia, and/or aberrant CDX2 expression were seen in 21.95 % (18/82) of the children, potentially identifying those who would benefit from closer surveillance and preventive dietary strategies. Biomarkers CDX2, EphB4, MMP3, and MIF may be useful in the work-up of pediatric gastritis.

Keywords

antral gastritis; biomarkers; child; Helicobacter pylori; Mexican; surveillance

Introduction

Chronic inflammation is a risk factor for carcinogenesis in several tissues, including the stomach[1, 2]. Inflammation is a well-coordinated response of the innate and adaptive immune systems following infection or injury[1]. Deregulation of the inflammatory response leads to unresolved inflammation and a pro-neoplastic microenvironment[1]. The tissue damage produced by high levels of phagocyte-generated reactive oxygen, nitrogen, and halogen species can cause mutations and cell death and play a key role in the carcinogenic process[2].

Chronic gastritis in children has multiple etiologies, including gastroesophageal reflux, food allergies, high intake of spicy food, acid peptic disease, non-steroidal anti-inflammatory drugs, and *Helicobacter pylori* infection. *H. pylori* infection increases the production of reactive oxygen and nitrogen species, and the gram-negative microaerophile confers nearly an eleven-fold increased risk of gastric cancer (GC)[3]. Infection with *H. pylori* is highly prevalent among socially and economically disadvantaged children. Age, overcrowding, number of siblings, and a low maternal education level increase infection risk[4-7].

Globally, GC is the fourth most common cancer and second highest cause of cancer mortality with nearly two-thirds of these deaths occurring in developing nations[3]. Although we seldom see GC in children, these issues are of keen interest in underdeveloped countries where *H. pylori* is highly prevalent. Gastric carcinogenesis is hypothesized to be a process involving a number of premalignant genetic and morphologic alterations of gastric mucosa. Busuttil and Boussioutas[3] outline the progression from normal stomach to gastritis, and intestinal metaplasia (IM). IM is considered a preneoplastic lesion, although it should be noted not all IM advances to dysplasia, the step previous to GC[8,9].

Given the important role of chronic inflammation in carcinogenesis, we sought to determine whether Mexican children with a pathologic diagnosis of chronic antral gastritis exhibited histologic markers associated with adult preneoplastic lesions. Secondly, since Caudal Type Homeobox 2 (CDX2) expression precedes the development of gastric preneoplastic lesions in the setting of IM, we sought to define the *H. pylori* status and expression of CDX2 in our cohort of children and compare them with Mexican and American adult cohorts. Finally, we seek a panel of candidate biomarkers to use routinely in gastric biopsies in pediatric populations with a high prevalence of *H. pylori* infection[7]. Therefore, we selected an immunohistochemical (IHC) protein profile involved in gastric carcinogenesis and progression: Caudal Type Homeobox 2 [10], Ephrin Type-B Receptor 4 (EphB4)[11-12], Matrix Metalloproteinase 3 (MMP3)[13-14], Macrophage Migration Inhibitory Factor (MIF)[15], p53

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(TP53 tumor ressor gene)[16], β-catenin, and E-cadherin[17-18]. Our ultimate goal is the identification of children with antral lesions who would benefit from closer follow up surveillance, preventive nutritional strategies, and health promotion activities.

Materials and Methods

Patients and Samples

This study was conducted with the approval of the Central Military Hospital and the Medical College of Wisconsin Institutional Review Board (IRB). Consecutive gastric antral biopsy samples were obtained from 82 Mexican children (Table 1) of middle socioeconomic status attending the Central Military Hospital in Mexico City the first 3 months of 1996 and 2009.

Patients presented with one or more of the following symptoms: chronic epigastric or abdominal pain, pyrosis, or gastrointestinal bleeding. Gastroesophageal junction, antrum, and duodenum biopsies were examined by an attending hospital pathologist. Our cases did not include autoimmune gastritis, chemical gastritis, primary bile reflux gastritis, inadvertent sampling of the gastroduodenal junction, or postoperative gastritis, and none had received *H. pylori* eradication therapy. The adult biopsies were used as controls to compare CDX2 and the gastritis criteria with the children's biopsy results. Thirty-five adult antral specimens were obtained from either Froedtert Hospital in Milwaukee, WI (n=14) or the Mexican Institute of Social Security (n=21) (Table 1).

Immunohistochemical Staining

Biopsy specimens were fixed in 10% buffered formalin and embedded in paraffin. Four micrometer thick sections were deparaffinized in xylene, hydrated in descending dilutions of ethanol, and exposed to heat-induced epitope retrieval. See Table 2 for details.

Pediatric CDX2 and p53 IHC staining was performed manually. IHC for β-catenin, E-cadherin, adult CDX2, EphB4, MIF, MMP3, and *H. pylori* was performed using reagents from the Dako Envision FLEX High pH kit and the Dako Autostainer Plus. Following pretreatment with Target Retrieval Solution (TRS pH 9.0), tissue was blocked with peroxidase-blocking reagent for 5 min, treated with PBS/BSA (EphB4, MIF, MMP3) for 30 min, incubated with primary antibody for 10 min (CDX2) or 30 min (β-catenin, E-cadherin, EphB4, MIF, MMP3) at room temperature, followed by 20 min horseradish peroxidase, 10 min DAB, and EnVision FLEX hematoxylin (Dako, Carpinteria, CA, US) counterstain.

Immunohistochemistry Analysis

The percentage of total gastric gland epithelial cells staining positive was determined (EphB4, MIF, MMP3). The samples were analyzed by a pathologist (ACM and/or ALG) blind to gastritis parameter scores or *H. pylori* infection status. To validate IHC, we stained various adult tissue specimens. These staining patterns were used as reference intensities for the gastric staining and scoring. β -catenin, E-cadherin, and CDX2 were scored as either 1 (low) or 2 (high) staining intensity. β-catenin expression was evaluated in membranous or nuclear location. Staining intensity for EphB4, MIF, and MMP3 was determined as: 0

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(none), 1 (mild), 2 (moderate), and 3 (strong). The most intense stain covering at least 10% of the biopsy was used as the intensity score. p53 was evaluated based on presence of nuclear positivity. Positive control tissues included: breast cancer (p53), colon (β-catenin, E-cadherin, EphB4), duodenum (CDX2), and liver (MIF, MMP3).

Four criteria of chronic gastritis—gastritis activity, atrophy, follicular pathology, and IM—were evaluated by a pathologist (ALG). Histological classification of all biopsies' H&E stains was done according to the Updated Sidney System[19].

Warthin-Starry stain was used for the detection of *H. pylori* in the U.S.A. adult samples. A modified Giemsa stain was used to detect *H. pylori* in the specimens from the Mexican children and adults. All pediatric samples that were *H. pylori* negative were confirmed with *H. pylori* IHC.

Statistical Analysis

Statistical analyses were performed using SAS 9.2 Statistical software. The correlation between the antibodies and gastritis parameters was measured by Spearman Rank-order test. The association between *H. pylori* status and gastritis criteria was investigated by Pearson Chi-square test. The association of antibody staining intensities with *H. pylori* infection status as well as the association of CDX2 with *H. pylori* infection status for children and adults was tested by Fisher's exact test. Significance was set at $p < 0.05$.

Results

The distribution of selected gastritis histopathology and immunohistochemistry variables in the pediatric cohort is shown in Table 3. Antral atrophy was seen in 7/82 (8.5%) and IM in 5/82 (6.1 %) by H&E staining (Figure 3). The IM was classified as Type I for 4/5 cases and type II for the fifth case[20]. Of the 7 children with atrophy, 6 had *H. pylori* + biopsies, while of the 5 children with IM, one was *H. pylori* +.

Expression of β-catenin and E-cadherin expression occurred in 96% and 100% of cases respectively and was membranous (Figure 1). There was no nuclear β-catenin staining. No biopsies exhibited p53 nuclear positivity. Six additional biopsies exhibited aberrant CDX2 expression without histologic evidence of IM (Figure 1), and CDX2 expression did not correlate with *H. pylori* infection. Expression of MMP3, EphB4, and MIF was present in 96%, 90% and 62 % of biopsies, respectively (Figure 2). There was a positive correlation between cases with EphB4 positivity and MMP3 and MIF positivity ($p<0.0001$).

Significant negative correlations were noted between the severity of gastritis activity and MMP3 ($p=0.0255$) and EphB4 ($p=0.0432$), as well as between atrophy and MMP3 percent positivity ($p=0.0370$).

H. pylori Positive and H. pylori Negative Children Groups

In children, *H. pylori* positive versus negative biopsies differed significantly with respect to atrophy (Chi-square 56.5744, Asymptotic Pr>Chi-Sq $p<0.0001$) and follicular pathology (Chi-square 11.3981, Asymptotic Pr>Chi-Sq $p<0.0007$), but not with respect to IM (Chi-

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square 1.2353, Asymptotic Pr>Chi-Sq p=0.2664). Membranous β-catenin intensity was significantly higher in biopsies from *H. pylori* positive patients compared with those from *H. pylori* negative patients (p=0.0026). In *H. pylori* positive biopsies, negative correlations were noted between MMP3 and atrophy (p=0.0484) and EphB4 and gastritis activity (p=0.0475). In *H. pylori* negative biopsies, MMP3 staining correlated negatively with atrophy (p=0.0256) and gastritis activity (p=0.0015).

CDX2 Expression: Child and Adult

CDX2 was positive in 6 (7%) of children's gastric biopsy specimens (3 *H. pylori* + and 3 *H. pylori* -), and exhibited no significant associations with any of the gastritis criteria. CDX2 expression was more frequent in adult biopsies than in children's biopsies regardless of infection status (p<0.0001), as well as within *H. pylori* + (p=0.0122) and *H. pylori* - (p<0.0001) adult cohorts. Adult CDX2 percent positivity showed significant positive correlations with both IM (p=0.0019) and atrophy (p=0.0433).

Discussion

We found that 18 of 82 antral biopsies from children had either atrophy, intestinal metaplasia (IM), and/or ectopic CDX2 expression. This is notable considering that IM and atrophy are relatively rare in other reported studies of pediatric gastritis[6,21, 22]. As CDX2 is expressed early in the IM progression pathway, ectopic antral CDX2 probably reflects pathologic changes leading to IM[3]. In transgenic mice, gastric expression of CDX2 alone can induce IM[25]. Children's biopsies had significantly less CDX2 positivity than adults' biopsies, regardless of infection status. Adults also showed a significant positive correlation of CDX2 with both IM and atrophy. In contrast, children's biopsies with CDX2 positivity exhibited no significant associations with these two gastritis parameters or with *H. pylori* status. Current literature suggests IM regression is rare. While elimination of *H. pylori* is associated with regression of gastric inflammation and atrophy, IM usually persists[23]. Since 80% of gastric carcinomas arise in the context of IM and its presence results in a two to six-fold increased risk for cancer development[23,26], the finding of CDX2 positivity in 7% of children warrants further exploration for its association with either *H. pylori* or other chronic gastritis etiologies.

As the regulatory mechanisms involved in triggering and maintaining gastric CDX2 expression are not entirely clear[27], we were interested in two recent papers describing novel CDX2 regulatory mechanisms relevant to this work. Barros *et al*. suggested an auto-regulatory CDX2 loop, which may have a major impact on the stability of human IM, possibly resulting in progression along the gastric carcinogenesis pathway[23]. Results from Camilo *et al*. provided a link between *H. pylori* infection and the Bone Morphogenetic Protein (BMP) pathway in the regulation of intestinal and gastric-specific genes that might be relevant for gastric IM[24]. It remains to be seen what CDX2 regulatory mechanisms participate in the absence of *H. pylori*[25-28].

Atrophy exhibited a strong correlation with *H. pylori* positive status in our children. Chronic atrophic gastritis has been considered a progressive disease worsened by *H. pylori* infection, use of non-steroidal anti-inflammatory drugs and proton pump inhibitors, and with the

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intake of carbonated drinks and fast food[29]. This is relevant to our cohort's diet and high consumption of soft drinks in Mexico—an average of 163 L per capita per year compared to 118L in the U.S.A.).[30]

The significant positive associations between EphB4, MMP3, and MIF ($p<0.0001$) suggest interaction between the EphB pathway which regulates the degradation of extracellular matrix proteins, cell adhesion proteins, and an inflammatory cytokine in the progression from chronic inflammation to carcinogenesis. EphB4 is part of the Eph (erythropoietin-producing hepatoma) receptor tyrosine kinase family regulating cell migration during embryonic development and adhesion and migration of cancer cells. It is fundamental for angiogenesis, vessel maturation, and pericyte recruitment[11]. It has been consistently found in most epithelial cancers, including gastric cancer[12]. The expression of EphB4 in 90% of antral samples is pertinent to the recently emerging unifying theme outlined in Wang *et al*., in which an evolving cancer cell may either directly eliminate the anti-migratory effects of the activated Eph receptors, or the Eph receptors aid in migration and invasion[31]. This novel concept is highly relevant in the setting of gastric carcinogenesis because epithelial cells with high expression of Ephs and ephrins could have a survival advantage and participate in the tumor progression selection[31]. The persistence of high EphB4 immunoreactivity in gastric glands in the scenario of IM and gastric atrophy raises questions about malignant progression, particularly if the high expression is not ameliorated by treatment[31,32].

MMP3 upregulation has also been associated with gastric carcinogenesis[13,14]. In the Economescu *et al*. study[13], MMP3 upregulation was associated with gastric tumor progression, while Rajkumar *et al*. [14] demonstrated MMP3 upregulation in gastric carcinomas but not in the adjacent non-neoplastic gastric mucosa. Moreover, MMP3 promoter polymorphisms (MMP3707 G/G and MMP3-1612 5A/6A) are potential independent predictors of gastric cancer risk development[33].

Macrophage migration inhibitory factor (MIF) is a multifunctional cytokine which plays important roles in inflammation and tumorigenesis. Polymorphisms such as MIF-173 and MIF-794-CATT have been associated with risk for severe chronic atrophic gastritis[34]. Mice studies have found both serum and gastric MIF immunoexpression progressively increase in *H. pylori*-induced gastritis, IM, and gastric cancer, and even in gastric injury due to non-steroidal anti-inflammatory drugs[35,36]. Fehlings *et al*. demonstrated that MIF suppression by *H. pylori* infected monocyte-derived dendritic cells enables immune evasion mechanisms, promoting the bacterium's persistence[37]. MIF knockout mice, on the other hand, did not develop gastritis after *H. pylori* infection—the inhibition of *H. pylori*-induced innate immune responses and Th1 -- mediated immune injury playing a probable role[38]. Given the uncertainty of the role of MIF in pediatric antral biopsies, this marker should be further explored in the evaluation of these specimens.

High membranous β-catenin expression was significantly associated with *H. pylori* positive biopsies, while E-cadherin expression was not associated with *H. pylori* status. Infection with *H. pylori* is associated with deregulated accumulation of nuclear β-catenin and promotes malignant transformation, while mutations of CTNNB1 genes occur early in GC development and contribute to gastric carcinogenesis[18,39]. The Wnt/ β-catenin pathway,

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among its many vital developmental roles, is also involved in the development of cancer and in supporting cadherin-mediated cell adhesion[40]. It will be of interest to expand this observation to larger cohorts, and especially with longitudinal follow-up. *H. pylori*-induced calpain activation results in cleavage of E-cadherin to produce a truncated form and induce relocalization of E-cadherin and β-catenin and inhibition of TLR2 prevented *H. pylori*-induced calpain activation and adherens junctions (AJ) disassembly[41]. O′Connor suggested *H. pylori* activates calpain via TLR2 to disrupt gastric epithelial AJ structure—in turn, the disruption of AJ structure favors severe disease[41].

The presence of atrophy, IM and CDX2 positive cells independent of *H. pylori* status suggest a need for more intense clinical surveillance in this pediatric cohort. Close follow-up should be indicated for children with CDX2 nuclear positivity even in the absence of intestinal metaplasia by H&E, high expression of EphB4 [31], and those with atrophy. Biopsies with atrophy should be checked for *H. pylori* by modified Giemsa, Warthin-Starry, or *H. pylori* antibodies. Moreover, since intestinal metaplasia rarely regresses and there are no published children's studies indicating the natural history of IM, follow-up is warranted.

Alongside the follow-up pathology and clinical surveillance, use of probiotics[42,43], avoiding the use of unnecessary antibiotics that wipe out beneficial bacteria[44], preventive dietary strategies i.e., diets high in vegetables and reduced intake of carbonated drinks and spicy food[29], along with health promotion activities (i.e., introduction of free, potable drinking-water fountains in schools and public spaces, educating parents on improving sanitation conditions, and nutritional guidance to mothers) should be encouraged.

The importance of our findings is limited by the absence of long-term clinical and pathologic follow-up. Expanding the number of cases and having access to sequential biopsies will allow us to define the progression of the preneoplastic lesions and the use of selected markers in future studies. Prospective studies of pediatric antral biopsies would benefit from differential gene expression profiling of key markers using laser microdissection a[45], and the use of DNA microarrays of preneoplastic lesions. Longitudinal studies plus molecular techniques will disclose the complex interplay of environmental and genetic factors in the development of gastric preneoplastic lesions in children.

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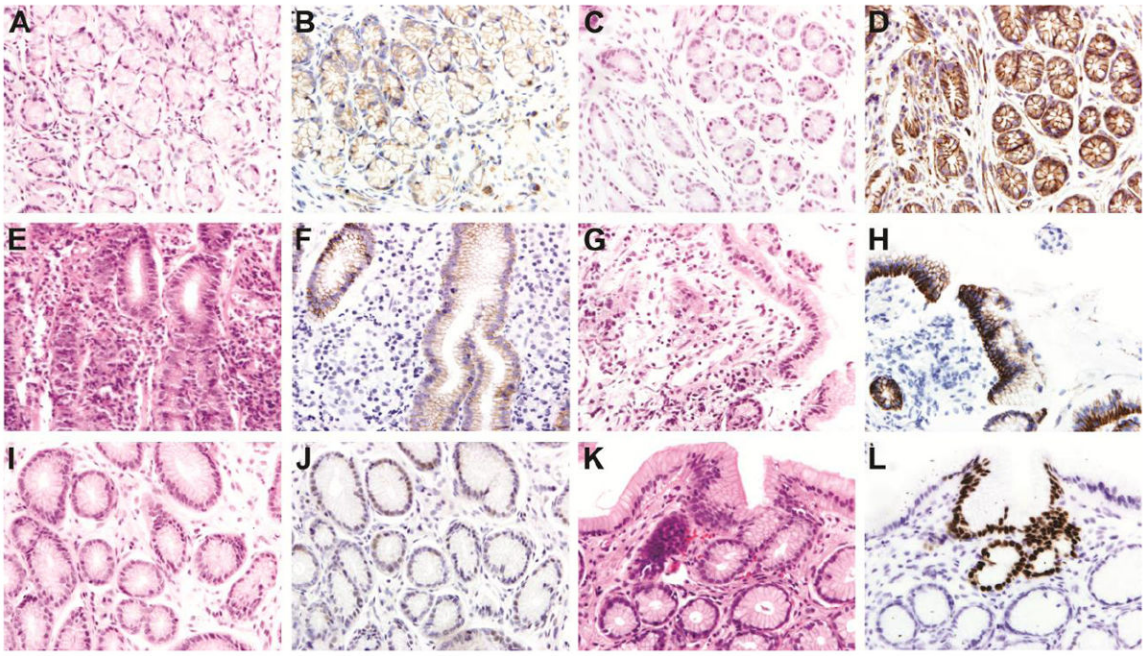

Figure 1. β-catenin, E-cadherin, & CDX2 (400x)

(A) Unremarkable antral biopsy from four-year-old girl, H&E. (B) β-catenin. Membranous positivity is weak with a patchy distribution. (C) 6 month old girl with *H. pylori* positive biopsy and focal glandular atrophy, H&E. (D) β-catenin. The membranous positivity is strong. There is no nuclear positivity. (E) Fifteen-year-old boy with active severe inflammatory response and *H pylori* positivity, H&E. Elsewhere in the biopsy there was focal atrophy and follicular pathology. (F) E-cadherin. Weak focal membranous immunoreactivity. (G) Fifteen-year-old-boy with abdominal pain and a finely granular gastric mucosa at endoscopy, H&E. Mild chronic inflammation and elsewhere follicular pathology. (H) E-cadherin. Strongly positive membranous staining. (I) Eight-year-old girl with an unremarkable, H. pylori negative antral biopsy, H&E. (J). CDX2. Positive weak nuclear staining in mostly dilated gastric glands. (K) Fifty-nine-year old with *H. pylori* positive biopsy and intestinal metaplasia, H&E. (L) CDX2. Nuclear immunoreactivity is strong in the area of intestinal metaplasia.

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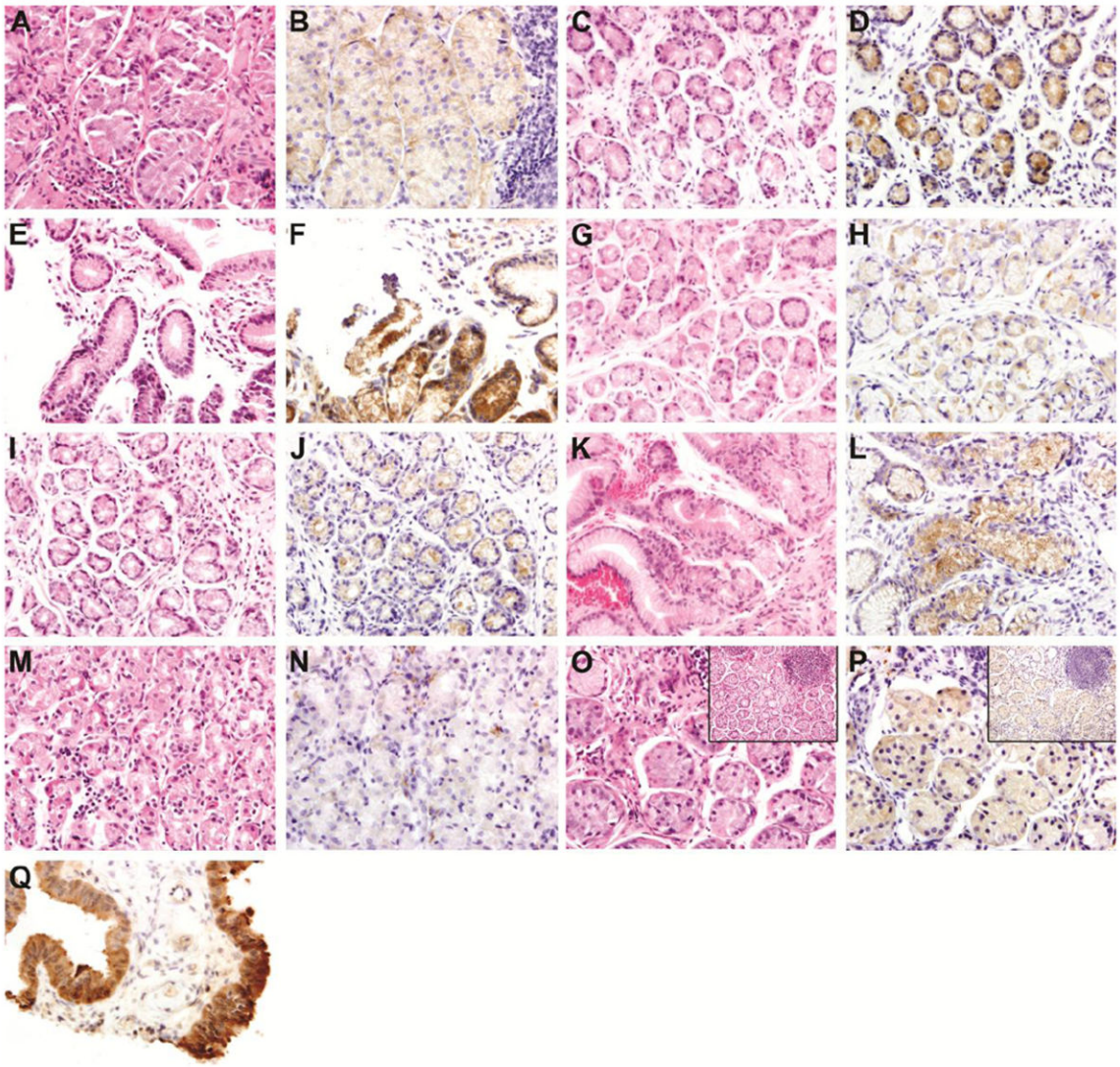

Figure 2. EphB4, MMP3, & MIF (400x)

(A) Eight-year-old girl with a history of abdominal pain and *H. pylori* positive biopsy. Moderately active gastritis, H&E. (B) EphB4. Weak immunoreactivity of EphB4 in gastric glands surrounded by focal severe chronic inflammatory infiltrates. Inset shows a hyperplastic follicle H&E × 200. (C) Nineteen month-old boy with a history of abdominal pain, normal gastric mucosa at endoscopy and negative *H. pylori*. Mild inflammation, H&E. (D) EphB4. Moderate immunoreactivity is seen in glands surrounded by mild inflammatory activity. (E) Eight year old girl, biopsy negative for *H. pylori*. Mild inflammatory activity, H&E. (F) EphB4. Strong EphB4 immunoreactivity is seen in glands surrounded by mild inflammation.

(G) Twelve year old girl with a history of chronic abdominal pain, mild gastritis by endoscopy and antral biopsy positive for *H pylori*. Mild inflammatory activity, H&E. (H) MMP3. Scattered glands with weak cytoplasmic staining. (I) Three year old boy with a clinical history of chronic abdominal pain, mild gastritis by endoscopy. and antral biopsy positive for *H. pylori*, H&E. There is mild inflammatory activity. (J) MMP3. A moderate

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number of gastric glands showed cytoplasmic immunoreactivity. (K) Seventeen-year-old girl with a normal endoscopy and positive *H. pylori.* Mild inflammatory activity, H&E. (L) MMP3. Strong immunoreactivity of gastric epithelial cells in association with moderate focal chronic inflammation. (M) Nine-year-old girl with *H. pylori* positive biopsy. Mild inflammation, H&E. (N) MIF. Scattered MIF-positive glands. (O) Eight-year-old girl with a history of abdominal pain and a granular antral biopsy at endoscopy. Moderate inflammation, H&E. Inset: hyperplastic follicle. (P) MIF. Moderate cytoplasmic MIF staining. (Q) MIF control with strong positivity.

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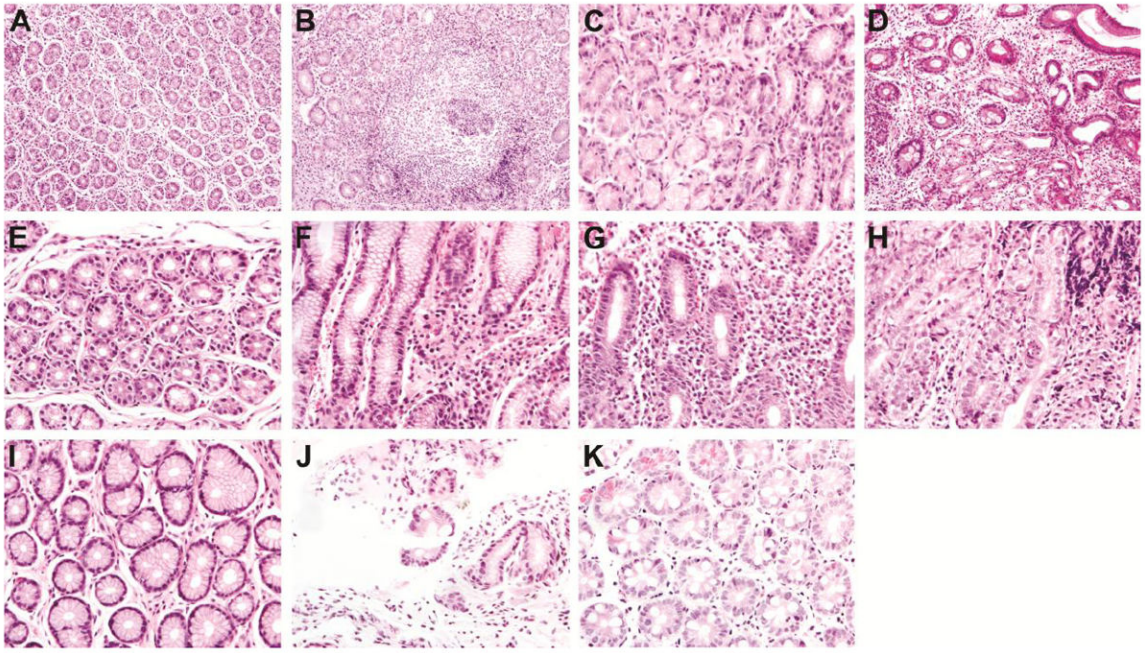

Figure 3. Representative pediatric gastric biopsies showing gastritis criteria

(A) Normal follicles. (B) Hyperplastic follicles. (C) Absent atrophy. (D) Atrophy present. (E) Absent inflammation. (F) Mild inflammation. (G) Moderate inflammation. (H) Marked inflammation. (I) Absent intestinal metaplasia. (J) Incomplete intestinal metaplasia. (K) Complete intestinal metaplasia.

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Table 1

Patient age, gender, and Helicobacter pylori status

Variables	Children (n: 82)	Adults (n: 35)
Age [mean years (range)]	8.1 (0.3 to 17)	66.3 (50 to 81)
Gender (female, male)	47, 35	18, 17
H. pylori (+, -)	36, 46	21, 14

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Table 2

Antibodies and pretreatment used

Antigen	Clone	Source	HIER*	Dilution
β-Catenin	β-Catenin-1	Dako	TRS pH 9.0	RTU**
CDX2 (children)	AMT28	Leica Microsystems	Citrate buffer pH 6.0	1/100
CDX2 (adults)	DAK-CDX2	Dako	TRIS pH 9	RTU
E-Cadherin	NCH-38	Dako	TRIS pH9	RTU
EphB4	3D7G8	InVitrogen	TRIS pH9	1/50
H. pylori	Rabbit	Dako	Citrate pH 6	RTU
MIF	D-2	Santa Cruz Biotech	TRIS pH 9	1:200
MMP3	10D6	R&D Systems	TRIS pH 9	1:50
P53	Pab 1801	Leica Microsystems	Citrate pH 6	1:500

* HIER:heat-induced epitope retrieval;

** RTU: Ready to use

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Table 3

Histopathologic and immunostain findings in pediatric cohort (n=82)

Variable	Score	n	%
Gastritis	0 (No Activity)	39	47.6
	1 (Mild)	30	36.6
	2 (Moderate)	6	7.3
	3 (Strong)	7	8.5
Atrophy *	No	75	91.5
	Yes	7	8.5
Follicular pathology *	none	70	85.4
	Mild	11	13.4
	Marked	1	1.2
Intestinal metaplasia *	No	77	93.9
	Yes	5	6.1
H pylori	Negative	46	56.1
	Positive	36	43.9
p53	Negative	82	100
CDX2	Low	76	92.7
	High	6	7.3
MIF	0	30	34.2
	1+	45	55.7
	2+	5	6.3
MMP3	0	3	3.8
	1+	15	19
	2+	14	17.7
	3+	32	59.4
EphB4	0	8	10.1
	1+	36	45.6
	2+	31	39.2
	3+	4	5.0
β-catenin, membranous	0	3	4.0
	1+	46	60.5
	2+	27	35.5
E-cadherin	1+	8	10.1
	2+	71	89.9

* Four criteria of chronic— gastritis gastritis activity, atrophy, follicular pathology, and intestinal metaplasia were evaluated according to the Updated Sidney System.