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Aberrant expression of *CDX2* in Barrett's epithelium and inflammatory esophageal mucosa

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Background. There have been no detailed reports directly comparing the expression of CDX1 with that of CDX2 in the inflammatory esophageal mucosa and Barrett's epithelium. The present study was designed to examine the expression of CDX 1/2 in inflammatory esophageal mucosa with or without Barrett's epithelium. ***Methods.*** The expression of *CDX1/2* genes was analyzed using the reverse transcriptase-polymerase chain reaction (RT-PCR) in 34 human esophageal biopsy specimens, and CDX2 expression was also evaluated immunohistochemically, using anti-human CDX2 monoclonal antibody. The biopsy specimens for RNA extraction were taken endoscopically from esophageal mucosa with mucosal break due to gastroesophageal reflux disease (GERD), Barrett's epithelium, and normal epithelium. The expressions of mucin markers (MUC2) and intestine-specific genes (sucrase-isomaltase, human defensin-5, alkaline phosphatase) were also comparatively analyzed. ***Results.*** CDX1/2 expression was not found in the normal esophageal mucosa. The prevalence of *CDX1/2* mRNA expression was significantly higher in the mucosa with Barrett's epithelium than in the mucosa without Barrett's epithelium. It is noteworthy, however, that the *CDX2* mRNA expression was initiated at the stage of esophagitis, when neither *CDX1* nor intestine-specific genes had emerged yet. In contrast to *CDX2*, *CDX1* was expressed only in Barrett's epithelium. Immunohistochemical study demonstrated strong and extensive nuclear immunoreactivity for CDX2 in Barrett's epithelium. Furthermore, fine granular cytoplasmic staining was also observed in the cytoplasm in Barrett's epithelium, as well as in inflammatory esophageal mucosa. ***Conclusions.*** We report here, for the first time, that CDX2 is expressed in patients with Barrett's epithelium and inflammatory esophageal mucosa. These findings imply that the expression of CDX2 may be an early event leading to the development of Barrett's esophagus.

Key words: Barrett's epithelium, CDX1, CDX2, gastroesophageal reflux disease

Introduction

The *CDX1* and *CDX2* genes are intestinal transcription factors that may be involved in the regulation of the proliferation and differentiation of intestinal epithelial cells. *CDX1/2* are members of the caudal-related homeobox gene family based on their sequence homology to the caudal gene of *Drosophila melanogaster*. The caudal gene is necessary for anteroposterior polarity during early *Drosophila* development.[1–4] CDX1/2 protein is predominantly expressed in the intestine and colon, but not in the normal epithelium of the esophagus and stomach through adulthood in humans and mice.[3,5–7]

Although Barrett's epithelium is classified into three types of columnar epithelia above the lower esophageal sphincter,[8] the most specific distinguishing observation of Barrett's epithelium is the presence of specialized columnar epithelium with a villiform surface, mucus glands, and intestinal-type goblet cells, devoid of the brush-border characteristic of absorptive epithelium ("incomplete form"of intestinal metaplasia). In addition to this type, there is a complete type of intestinal metaplasia with brush-border and Paneth's cells, devoid of a villiform surface.

Many gene products, such as intestinal-type alkaline phosphatase (ALP);[9,10] the well characterized brush-border enzyme, sucrase-isomaltase (SI),[10,11] which is expressed in 76% of Barrett's esophagus;[12] human defensin-5 (HD),[13–15] which is expressed predominantly

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in Paneth's cells; and mucus-secreting goblet cell-mucin marker (MUC2),[16,17] are associated with gastric and esophageal intestinal metaplasia.

Barrett's mucosa is often associated with chronic gastroesophageal reflux disease (GERD),[18–20] but genetic events predisposing to Barrett's mucosa are not well documented. We have reported that the expression of *CDX2* precedes those of *CDX1*, *SI*, other intestine-specific genes (*HD*, *ALP*) and *MUC2* during the progression of gastric intestinal metaplasia.[21] Furthermore, we confirmed the aberrant CDX2 expression in chronic gastritis and intestinal metaplasia using immunohistochemistry.[22] Our findings imply that the expression of *CDX2* is initiated at the stage of chronic gastritis, and the expression of *CDX2* may not be the result of, but the trigger for, the chronic gastritis/metaplasia transition in the stomach. Furthermore, we generated a transgenic mouse in which intestinal metaplasia was induced by expressing *CDX2* in the stomach.[23] Therefore, we consider that *CDX2* expression may play a critical role in the development of intestinal metaplasia.

A previous investigation showed that *CDX1* was also expressed in the intestinal metaplasia of the esophagus, stomach,[24,25] and bile duct.[25] However, *CDX2* expression has not been studied comparatively with that of *CDX1*, nor with that of intestine-specific marker genes.

Accordingly, we focused on specialized columnar epithelium and examined the expression patterns of *CDX1/2* in inflammatory esophageal epithelium and Barrett's epithelium, in order to gain insight into the role of these homeotic genes in the progression of Barrett's epithelium.

Subjects and methods

Ethical approval

The study was approved by the Ethics Committee of the Jichi Medical School, Japan. Written informed consent was obtained from all patients.

Human esophageal tissue samples

We studied 34 patients who underwent routine upper endoscopy with biopsies at the Department of Gastroenterology, Jichi Medical School. Biopsy samples were immediately snap-frozen in liquid nitrogen and then stored at −80°C until processed.

Endoscopy with biopsy

Barrett's epithelium was defined endoscopically as any tongues of pink mucosa and/or circumferential columnar-appearing mucosa proximal to the esophagocardiac junction (ECJ). The ECJ was determined endoscopically, using the definition of the ECJ as the distal end of the fine longitudinal vessels recommended by Hoshihara et al.[26]

Short-segment Barrett's esophagus (SSBE) and long-segment Barrett's esophagus (LSBE) were defined as a length of less than 3 cm and a length of 3 cm or more 3 cm, respectively, of columnar epithelium above the SCJ at endoscopy.[27,28] Endoscopic assessment of GERD was performed using the Los Angeles (LA) classification.[29]

Diagnosis of Barrett's epithelium

Features of Barrett's epithelium were judged based on molecular findings. Barrett's epithelium (specialized columnar epithelium) was judged to be present when there was expression of more than one of the gene markers for intestinal metaplasia (*HD*, *ALP*, and *MUC2*), in addition to *SI* mRNA being detected.

In all patients, one biopsy specimen for RNA extraction was taken endoscopically from the esophageal mucosa proximal to the ECJ, with or without mucosal break, or from Barrett's epithelium.

In addition, in 15 patients, for comparative study with immunohistochemistry, one set of two side-by-side biopsy specimens was taken endoscopically from normal esophageal epithelium, inflammatory esophageal mucosa with mucosal break due to gastroesophageal reflux disease (GERD), and from Barrett's epithelium. RNA extraction was performed on one of the two biopsy samples, while the other sample was analyzed histologically (hematoxylin-and-eosin stain) and immunohistochemically. Biopsy specimens for histogical analysis were fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. Esophageal specimens were evaluated for the presence or absence of histological intestinal metaplasia and esophagitis.

Immunohistochemistry

The sections used for CDX2 immunohistochemistry were paraffin-embedded sections that were deparaffinized in xylene and treated with 3% hydrogen peroxide in methanol for 5 min to block endogenous peroxidase. The sections were immersed in citrate buffer (10 mM, pH 6.0) and heated for 20 min at 120°C in an autoclave. After the heating, the specimens were cooled for 60 min at room temperature. After incubation with blocking reagent (Dako Japan, Kyoto, Japan) for 10 min to eliminate non-specific staining, the sections were incubated with CDX2 monoclonal antibody to anti-human CDX2 protein (diluted 1:100; BioGenex, San Ramon, CA, USA) in a moist chamber overnight at

4°C. This CDX2 antibody reacts with a conserved epitope of the 40-kDa human CDX2 protein, according to the manufacturer. Then, the sections were incubated with Dextran polymer system/peroxidase (EnVision+; Dako Japan) for 90 min at room temperature. The color of immunostaining was developed with diaminobenzidine solution for 6–8 min, and the sections were counterstained with hematoxylin. The biopsy specimens of gastric intestinal metaplasia served as positive controls. For the negative control, sections were incubated with normal mouse IgG1, and no immunoreactivity was observed.

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Specific primers were designed for the *CDX1/2*, mucin marker (*MUC2*), and the intestinal metaplasia-associated antigenic molecules (*SI*, *HD*, and *ALP*). The primers used are listed in Table 1. The primer pairs for *CDX1/2* were designed to be located in different exons of the respective genes to exclude the effect of contamination by genomic DNA. Total RNA was isolated from tissues with Isogen (Nippon Gene, Tokyo, Japan), according to the protocols recommended by the manufacturer. Two micrograms of total RNA was reverse transcribed with random nanomers and reverse transcriptase (ReverTraAce; Toyobo, Osaka, Japan) following the conditions of the manufacturer.

The template cDNAs were amplified with Taq polymerase in the presence of the primer set. The thermocycling parameters used in the PCR were as follows: denaturation, 30 s at 94°C; annealing, 30 s at 54°C (63°C for *CDX1*, 60°C for *CDX2*); and extension, 30 s at 72°C. These reactions were repeated for 35 cycles. The PCR products were electrophoresed through a 2.0% agarose gel and stained with ethidium bromide. Similarly, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as an internal control. We confirmed the nucleotide sequences of the RT-PCR products by direct sequencing (data not shown).

Statistical analysis

Fisher's exact test was used to assess differences in the frequency of *CDX1/2* expression among the various groups shown in the contingency tables. A computed two-tailed *P* value of less than 0.05 was regarded as indicating statistical significance.

Results

Clinical and histological characteristics

Clinicopathological findings of the subjects are summarized in Table 2. The mean age of the patients was 54.7 years (range, 34–77 years), and the ratio of men to women was 16:18.

The patients were classified into three groups. Five patients who showed normal ECJ endoscopically and histologically were assigned to group N (specimens 30–34; mean age 55.2 years; men/women, 3:2). Fourteen patients assessed as having intestinal metaplasia, based on molecular findings, were assigned to the Barrett's epithelium group (specimens 1–14; mean age, 57 years; men/women, 5:9). All Barrett's epithelium was defined as SSBE at endoscopy. Fifteen patients assessed as lacking intestinal metaplasia, based on molecular findings, who showed esophagitis endoscopically were assigned to the GERD group (specimens 15–29; mean age, 52.4 years; men/women, 8:7). Endoscopic findings of GERD ranged from A to C, using the LA classification.

RT-PCR analysis

All the results of RT-PCR are listed in Table 2 and shown in Fig. 1. None of the intestinal gene markers was expressed in group N subjects. Neither *CDX1* nor *CDX2* was detectable (0/5) in the esophageal mucosa of group N patients.

The prevalence of *CDX1* mRNA expression in the esophageal mucosa was significantly higher in the mucosa with intestinal metaplasia than in the mucosa

Table 1. Primer pairs used in polymerase chain reactions (PCRs)

	Primer pairs	
Genes	Sense (5′ to 3′)	Antisense (5′ to 3′)
CDX1	AGCCGTTACATCACAATC	GAGACTCGGACCAGACCT
CDX2	GAGCTGGAGAAGGAGTTT	GGTGACGGTGGGGTTTAG
Sucrase	TGGCAAGAAAGAAATTTAGTGGA	TTATTCTCACATTGACAGGATC
Defensin-5	ATGAGGACCATCGCCATCCT	TCAGCGACAGCAGAGTCTGTAG
ALP	TGCAGGGGCCCTGGGTG	GCGTAGGTGCCGGCTGG
MUC2	ACAACTACTCCTCTACCTCCA	GTTGATCTCGTAGTTGAGGCA
GAPHD	CCACCCATGGCAAATTCCATGGCA	TCTAGACGGCAGGTCAGGTCCACC

Sucrase, sucrase-isomaltase; *defensin-5*, human defensin-5; *ALP*, alkaline phosphatase; *MUC2*, mucin marker; *GAPDH*, glyceraldehyde-3-phosphate-dehydrogenase

Table 2. Summary of gene expression in 34 samples

Case no.	Age (years)/sex	Endoscopic findings	Reverse transcriptase (RT)-PCR					
			CDX1	*CDX2*	*MUC2*	*Sucrase*	*Defensin*	*ALP*
Barrett's epithelium (n = 14)								
1	45/M	SSBE	+	+	+	+	+	+
2	53/F	SSBE	+	+	+	+	−	−
3	44/F	SSBE	−	+	+	+	−	−
4	53/F	SSBE	+	+	+	+	+	+
5	70/M	SSBE	−	+	+	+	−	−
6	43/F	SSBE	−	+	+	+	−	−
7	64/F	SSBE	+	+	+	+	+	+
8	48F	SSBE	−	+	+	+	−	−
9	56/F	SSBE	−	+	+	+	+	+
10	71/M	SSBE	+	+	−	+	+	+
11	47/F	SSBE	+	+	+	+	−	−
12	61/M	SSBE	+	+	+	+	−	−
13	66/M	SSBE	−	+	−	+	+	+
14	77/F	SSBE	+	+	−	+	+	−
GERD (n = 15)								
15	39/F	LA: A	−	+	−	−	−	−
16	76/M	LA: B	−	+	−	+	−	−
17	62/M	LA: A	−	−	−	−	−	−
18	67/F	LA: C	−	+	−	−	−	−
19	58/M	LA: B	−	+	−	−	−	−
20	64/M	LA: A	−	+	−	−	−	−
21	75/F	LA: C	−	−	−	−	−	−
22	44/F	LA: C	−	+	+	−	−	−
23	34/F	LA: B	−	−	−	−	−	−
24	50/M	LA: A	−	−	+	−	−	−
25	58/M	LA: A	−	+	−	−	−	−
26	37/F	LA: A	−	+	−	+	−	−
27	42/F	LA: A	−	+	−	−	−	−
28	38/M	LA: B	−	+	−	+	−	−
29	43/M	LA: A	−	−	−	−	−	−
Group N (n = 5)								
30	43/M	Normal	−	−	−	−	−	−
31	36/M	Normal	−	−	−	−	−	−
32	65/F	Normal	−	−	−	−	−	−
33	56/F	Normal	−	−	−	−	−	−
34	76/M	Normal	−	−	−	−	−	−

Sucrase, sucrase-isomaltase; ALP, alkaline phosphatase; SSBE, short-segment Barrett's esophagus; LA, Los Angeles classification; GERD, gastroesophageal reflux disease

without intestinal metaplasia (57% [8/14] vs 0% [0/15]; $P < 0.001$) (Fig. 2).

The prevalence of *CDX2* mRNA expression in the esophageal mucosa was also significantly higher in the mucosa with intestinal metaplasia than in the mucosa without intestinal metaplasia (100% [14/14] vs 67% [10/15]; $P < 0.001$) (Fig. 2).

Coexpression of *CDX1* and *CDX2* was observed in 57% (8/14) of the Barrett's epithelium. It is of note that the expression of *CDX2* emerged at the stage of esophagitis without expression of *CDX1* or gene markers for intestinal metaplasia (Fig. 2). In contrast to *CDX2*, *CDX1* was expressed only in Barrett's epithelium.

Immunohistochemistry

No immunoreactivity for CDX2 was observed in normal esophageal epithelium (Fig. 3).

Immunohistochemical study demonstrated strong nuclear immunoreactivity for CDX2 in an extensive area of Barrett's epithelium (Fig. 4). Furthermore, fine granular cytoplasmic staining was also observed in Barrett's epithelium, as well as in inflammatory esophageal mucosa, including both squamous mucosa and submucosal glands (Figs. 4, 5, 6). These staining patterns were not detected in the negative controls, or in the normal esophageal mucosa (Fig. 3, Table 2). The concordance rate between the histological presence

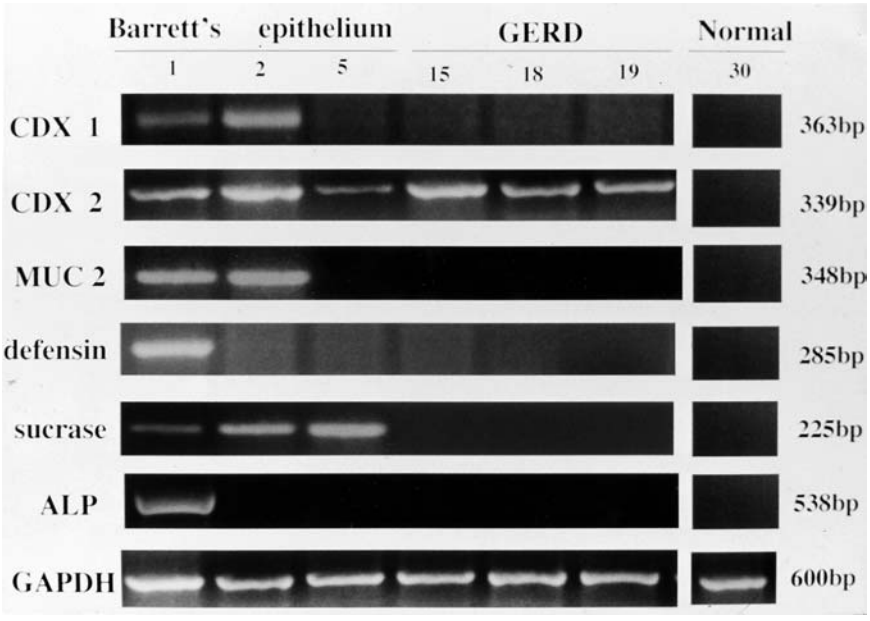

Fig. 1. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *CDX1/2*, mucin marker (*MUC2*), and intestinal metaplasia-associated antigenic molecules (human defensin-5 [*HD*], sucrase-isomaltase [*SI*], alkaline phosphatase [*ALP*]). *Left*, Genes; *right*, sizes of the PCR products. *Lane numbers* corresponds to Table 2 numbers. The results are summarized in Table 2. *GERD*, Gastroesophageal reflux disease; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase

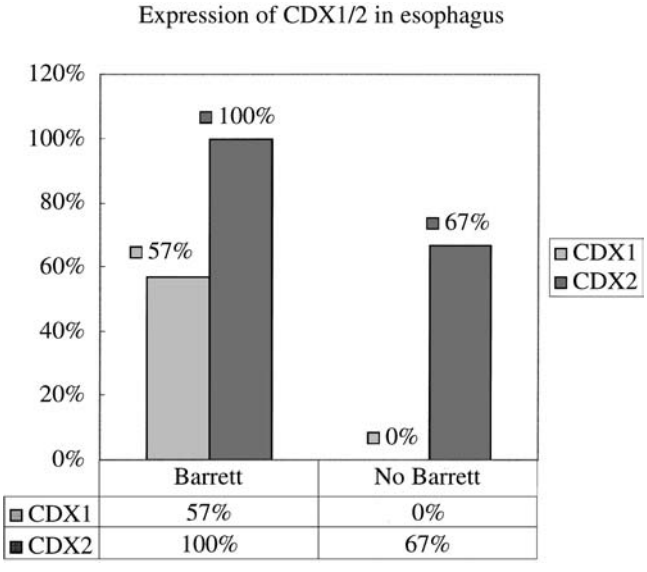

Fig. 2. Prevalence of *CDX1/2* expression in the esophageal mucosa. Expression of *CDX2* emerged in the esophageal mucosa without expression of *CDX1* and gene markers for intestinal metaplasia. The prevalence of *CDX1/2* mRNA expression was significantly higher in the mucosa with Barrett's epithelium than in the mucosa without Barrett's epithelium

of intestinal metaplasia (hematoxylin-and-eosin stain) and that diagnosed based on molecular findings was 87% (Table 3). The concordance rate between the presence of CDX2 expression determined by RT-PCR and immunohistochemical positivity was 100% (Table 3).

Discussion

The intestine-specific transcription factors CDX1 and CDX2 are important in the early differentiation and maintenance of intestinal epithelial cells during gastrointestinal development.[30,31]

In intestinal metaplasia, gastric and esophageal epithelial cells undergo changes that transform the cells into different phenotypes. The sequence of genetic events during the progression from normal epithelium to intestinal metaplasia is still unclear.

Many gene products, such as *ALP*, *SI*, *HD*, and *MUC2*, are expressed in intestinal metaplasia. It has been proposed that *CDX1* may play an important role in this transdifferentiation.[24] Epithelial cells in intestinal metaplasia of the gastric mucosa express the CDX1 protein, whereas normal gastric mucosa adjacent to areas of intestinal metaplasia has been immunohistochemically shown not to express CDX1.[24,25]

However, in addition to CDX1, the homologous transcriptional factor, CDX2, may also participate in this process.

Nevertheless, there has been no report about the detailed time sequence, i.e., when and how these gene expressions are evoked during the process of intestinal metaplasia. This study analyzed the complex patterns of expression of *CDX1* and *CDX2* during the development of Barrett's epithelium.

The *CDX1/2* expression rates appeared to be associated with the transition from GERD to Barrett's esophagus. In contrast to *CDX1*, *CDX2* was already expressed in inflammatory esophageal mucosa with-

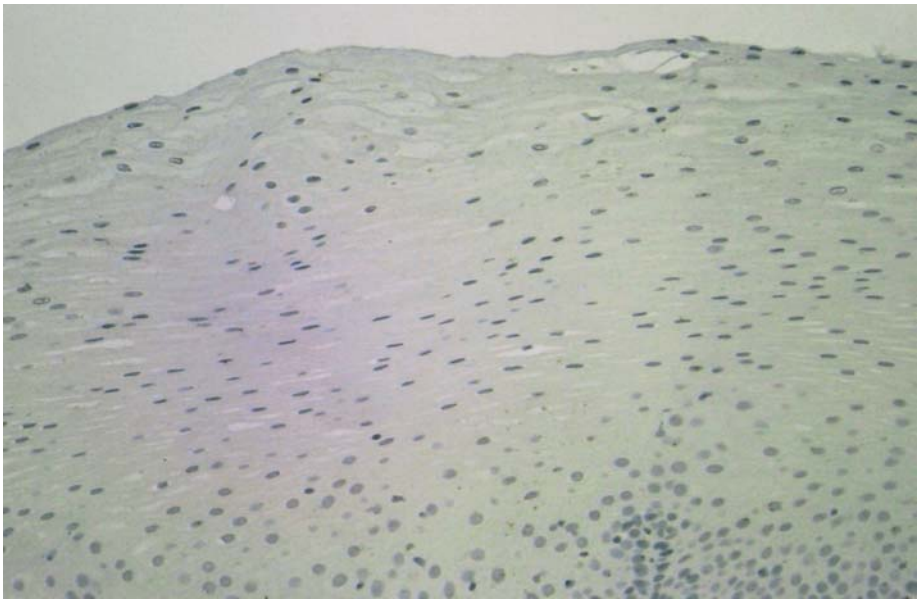

Fig. 3. Immunohistochemical study for CDX2 protein in normal esophageal epithelium. No immunoreactivity for CDX2 was observed in normal epithelium. × 200

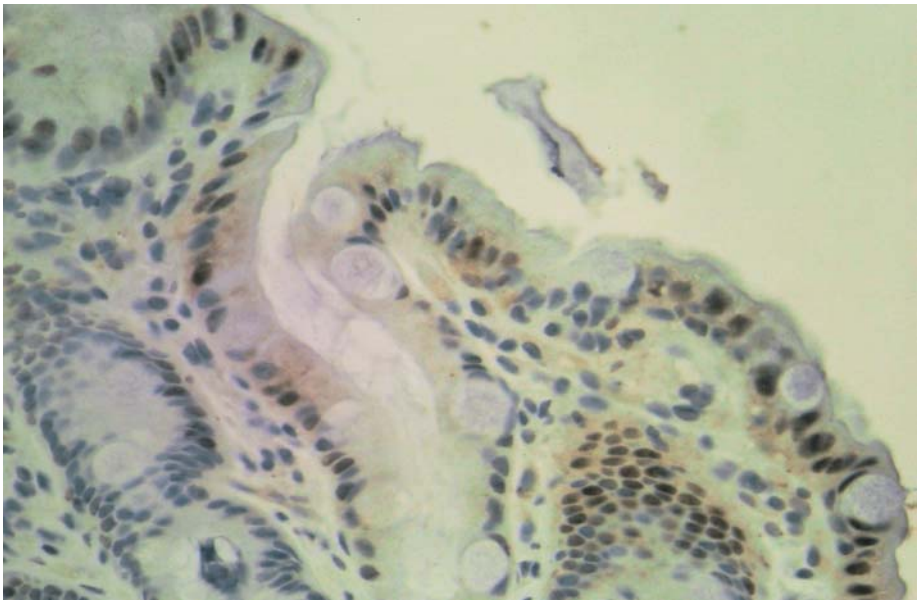

Fig. 4. Immunohistochemical demonstration of CDX2 protein in Barrett's epithelium. Strong nuclear immunoreactivity for CDX2 was observed in metaplastic glands. × 500

out the expression of gene markers for intestinal metaplasia.

The sequential pattern of gene expression demonstrated in the present study accorded with the scenario of the interaction among the intestine-specific genes in vitro. Our data show that the expression of *CDX2* occurred in the absence of *CDX1*, other intestine-specific genes (*SI*, *HD*, *ALP*), and *MUC2*. This pattern is consistent with the result that *CDX2* expression in Caco-2 cells induces the expression of *SI* and lactase-phlorizin hydrolase, markers of intestinal differentiation in vitro.[32] Both *SI* and lactase-phlorizin hydrolase promoters are activated by Cdx proteins.[3,32–34] Functional studies have also shown *CDX2* to regulate intestine-specific gene transcription in vivo, as evidenced by binding to several intestine-specific promoters and the activation of transcription.[35–37] Our finding implies that the expression of *CDX2* may not be the result of, but the trigger for, the development of intestinal metaplasia.

A set of two separate biopsy specimens for RNA extraction and histological examination may not be optimal for the detection and analysis of intestinal metaplasia,[38] because intestinal metaplasia is multifocal, and the possibility cannot be denied that sampling error may

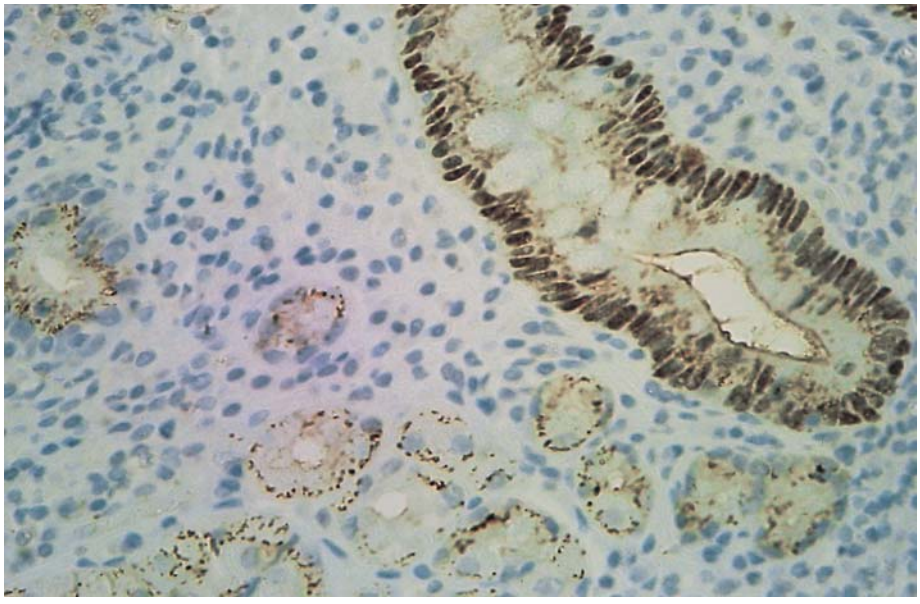

Fig. 5. Immunohistochemical demonstration of CDX2 protein in Barrett's epithelium. Strong nuclear immunoreactivity for CDX2 was observed in metaplastic glands, and cytoplasmic immunoreactivity for CDX2 was seen in submucosal glands. × 500

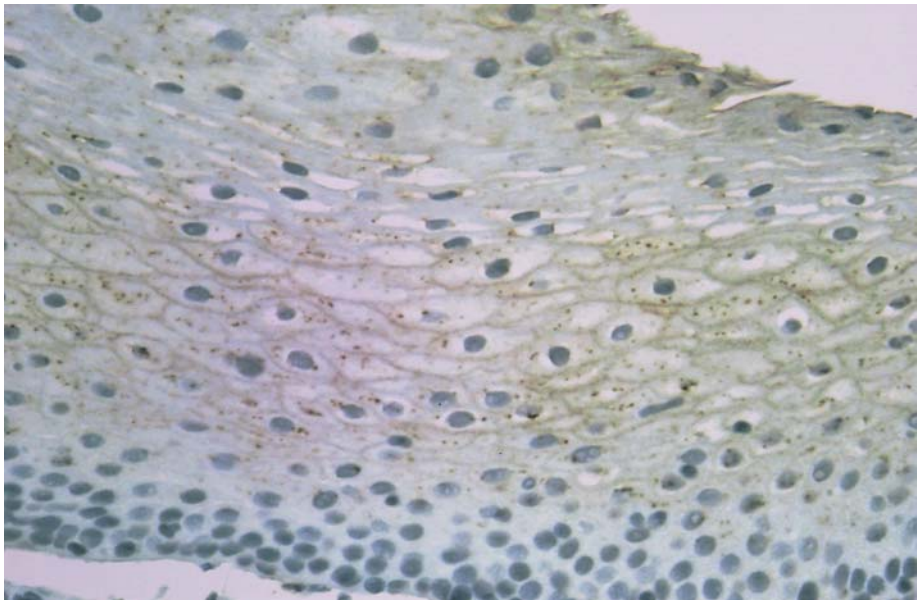

Fig. 6. Inflammatory esophageal squamous mucosa, characterized by fine granular cytoplasmic immunoreactivity for CDX2. × 200

ensue even if two adjacent biopsy samples are taken side by side.

Consequently, we characterized the specimens based on molecular findings and correlated *CDX1/2* expression with gene markers for intestinal metaplasia. As a result, the concordance rate between the histological presence of intestinal metaplasia and that diagnosed based on molecular findings was 87%. The concordance rate between the presence of *CDX2* expression, determined by RT-PCR, and immunohistochemical positivity was 100%. These results suggest that the *CDX2* expression rate in esophagus is high at the stage of esophagitis.

The sequential pattern of the relative expression of *CDX1/2* in metaplastic lesions may hold true across the differences in organs, between the esophagus and stomach. Namely, the sequential pattern of the expression of *CDX1/2* in the development of Barrett's epithelium is the same as that seen in the development of gastric intestinal metaplasia. In chronic gastritis, *CDX2* was expressed in the antral and fundic mucosa in the absence of expression of *CDX1* and gene markers for intestinal metaplasia (*SI*, *HD*, *ALP*, and *MUC2*) and hence, the expression of *CDX2* precedes those of *CDX1* and these intestine-specific genes during the progression of intestinal metaplasia.[21] Furthermore,

Table 3. Summary of histology, RT-PCR, and immunohistochemistry (IHC) for CDX2 in 15 samples

Case number[a]	Histology Intestinal metaplasia	CDX2 expression	
		RT-PCR	IHC
Barrett's epithelium (n = 5)			
1	+	+	+ (Nuclear)
2	+	+	+ (Nuclear)
3	−	+	+ (Cytoplasmic)
4	+	+	+ (Nuclear)
5	−	+	+ (Cytoplasmic)
GERD (n = 5)			
15	−	+	+ (Cytoplasmic)
16	−	+	+ (Cytoplasmic)
17	−	−	−
18	−	+	+ (Cytoplasmic)
19	−	+	+ (Cytoplasmic)
Group N (n = 5)			
30	−	−	−
31	−	−	−
32	−	−	−
33	−	−	−
34	−	−	−

[a] Case number corresponds to Table 2 number

we have confirmed the aberrant *CDX2* expression in chronic gastritis and intestinal metaplasia using immunohistochemistry.[22]

Barrett's epithelium is presumed to be the result of chronic inflammation caused by the gastric and duodenal juice, including bile, that flows back into the esophagus, whereas, it is presumed that gastric metaplasia is the terminal state of chronic gastritis caused by *H. pylori*. Therefore, any inflammation, irrespective of the cause, may play an important role in the induction of *CDX2* expression in the initiation of intestinal metaplasia in the esophageal and gastric mucosa.

In the GERD group, the expression of MUC2 and sucrase was positive in a few cases (cases 16, 22, 24, 26, and 28 in Table 2) by RT-PCR. These findings may result from the contamination of metaplastic cells. So, we analyzed CDX2 expression immunohistochemically to determine the precise localization of the CDX2 protein in the inflammatory esophageal mucosa and Barrett's esophagus. As a result, the immunohistochemical study demonstrated strong nuclear immunoreactivity for CDX2 in Barrett's epithelium. In contrast, perinuclear immunoreactivity for CDX2 was detected in the inflammatory esophageal mucosa, including both squamous mucosa and submucosal glands. During the progression from GERD to Barrett's esophagus, the localization of CDX2 protein may shift from cytoplasm to nucleus. The genetic mechanisms and candidate factors involved in this process should be explored in future. These data will provide insight into abnormal gene expression in the esophagitis/Barrett's esophagus transition.

It cannot be concluded that *CDX1/2* expression is the sole cause of intestinal metaplasia, based on the data shown here. However, we generated a transgenic mouse in which intestinal metaplasia was induced by expressing *CDX2* in the stomach.[23] Therefore, we consider that *CDX2* expression may play a critical role in the development of intestinal metaplasia.

In conclusion, we demonstrated here that the CDX2 homeodomain protein was ectopically overexpressed in Barrett's epithelium and inflammatory esophageal mucosa. These findings suggest that the expression of *CDX2* may be the crucial event leading to the progression of Barrett's esophagus, and that *CDX2* expression precedes that of *CDX1*, *SI*, other intestine-specific genes (*HD*, *ALP*), and *MUC2*.

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