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Expression of homeobox gene *CDX2* precedes that of *CDX1* during the progression of intestinal metaplasia

Akashi Eda, Hiroyuki Osawa, Ichiro Yanaka, Kiichi Satoh, Hiroyuki Mutoh, Ken Kihira, and Kentaro Sugano

Department of Gastroenterology, Jichi Medical School, Yakushiji, Kawachi, Tochigi 329-0438, Japan

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Background. The *CDX1* and *CDX2* genes are intestinal transcription factors that may be involved in the regulation of proliferation and differentiation of intestinal epithelial cells. There have been no detailed reports directly comparing the expression of *CDX1* with that of *CDX2* in chronic gastritis and intestinal metaplasia. Accordingly, we examined the expression of *CDX1/2* and its association with the expression of other intestinal metaplasia-associated genes during the development of intestinal metaplasia. ***Methods.*** The expression of *CDX1/2* genes was analyzed, using the reverse transcriptase-polymerase chain reaction, in 44 human gastric tissue samples obtained endoscopically. The expressions of mucin markers (MUC2, MUC5AC), intestine-specific genes (sucrase-isomaltase, human defensin-5, alkaline phosphatase), a gene marker for fundic gland area (H^+/K^+ATPase β subunit), and a gene for entire gastric glands (pepsinogen C) were also comparatively analyzed. ***Results.*** There was no expression of *CDX1/2* in gastric mucosa not infected by *Helicobacter pylori*. The prevalence of *CDX1* mRNA expression was significantly higher in mucosa with intestinal metaplasia than in mucosa without intestinal metaplasia. It is noteworthy that *CDX2* was expressed in the antral and fundic mucosa in the absence of the expression of *CDX1* and gene markers for intestinal metaplasia. ***Conclusions.*** The expression of *CDX2* precedes those of *CDX1*, sucrase-isomaltase, other intestine-specific genes (human defensin-5, alkaline phosphatase), and MUC2 during the progression of intestinal metaplasia. These findings imply that the expression of *CDX2* may trigger the initiation and development of intestinal metaplasia.

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Reprint requests to: A. Eda

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Introduction

The *CDX1* and *CDX2* genes are intestinal transcription factors that may be involved in the regulation of proliferation and differentiation of intestinal epithelial cells. *CDX1* and *CDX2* 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] The *CDX1/2* protein is predominantly expressed in the intestine and colon, but not in the normal epithelium of the stomach, through adulthood in humans and mice.[5–7]

Many gene products, such as the intestinal enzyme, intestinal-type alkaline phosphatase (ALP);[8,9] the well characterized brush-border enzyme, sucrase-isomaltase (SI);[9,10] human defensin-5 (HD),[11–13] which is expressed predominantly in Paneth's cells; and mucus-secreting goblet cell-mucin markers (MUC2),[14,15] are associated with gastric intestinal metaplasia.

A previous investigation showed that *CDX1* was also expressed in intestinal metaplasia of the esophagus, stomach,[16,17] and liver.[17] In addition to *CDX1*, a homologous transcriptional factor, *CDX2*, may participate in this process. *CDX1* has been studied more extensively than *CDX2*.

However, there have been no detailed reports that directly compared the expression of *CDX1* with that of *CDX2* in chronic gastritis and intestinal metaplasia, nor have there been any reports on the association of the expression of these genes with various types of intestinal metaplasia.

Accordingly, we examined the expression patterns of *CDX1/2* in human gastric epithelium, in order to gain

Table 1. Primer pairs used in PCR reactions

Genes	Primer pairs	
	Sense (5′ to 3′)	Antisense (5′ to 3′)
CDX1	CGGGCACACCGTCCTCGCCCG	CATTGGAGAGGAGGTGGCCAGG
CDX2	CGGCTGGAGCTGGAGAAGG	TCAGCCTGGAATTGCTCTGC
Sucrase	TGGCAAGAAAGAAATTTAGTGGA	TTATTCTCACATTGACAGGATC
Defensin-5	ATGAGGACCATCGCCATCCT	TCAGCGACAGCAGAGTCTGTAG
ALP	TGCAGGGGCCCTGGGTG	GCGTAGGTGCCGGCTGG
MUC2	ACAACTACTCCTCTACCTCCA	GTTGATCTCGTAGTTGAGGCA
MUC5AC	CTCCCACCCATCTGCTACAA	TGTGGTGAAGGTGGTCTGGG
$H^{+}/K^{+}ATPase$	ATGGCGGCTCTGCAGGAGAA	CGTGGAGAGTCTGTGTGACG
Pepsinogen C	ATGAAGTGGATGGTGGTGGT	GGTTGAAGCGGGAGTGACT
GAPDH	CCACCCATGGCAAATTCCATGGCA	TCTAGACGGCAGGTCAGGTCCACC

PCR, Polymerase chain reaction, sucrase, sucrase-isomaltase; defensin-5, human defensin-5; ALP, alkaline phosphatase; $H^{+}/K^{+}ATPase$, H^{+}/K^{+}ATPase β subunit

insight into the role of these homeotic genes in the progression of intestinal metaplasia.

Patients, materials, and methods

Ethical approval

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

Human gastric tissue samples

We studied 31 patients who underwent routine upper endoscopy with biopsies at the Department of Gastroenterology, Jichi Medical School. The mean age of the patients was 58 years (range, 34–77 years) and the ratio of men to women was 18:13. Biopsy samples were immediately snap-frozen in liquid nitrogen and then stored at −80°C until processed.

Endoscopy with biopsy

We examined biopsy specimens from the antrum and/or corpus (site A, the greater curvature of the mid-antrum within 2 cm from the pylorus; site F, the greater curvature of the middle body) for RNA extraction. Five patients who showed normal mucosa endoscopically and histologically were assigned to group N (normal). Twenty-six *Helicobacter pylori*-positive patients who were histologically diagnosed with chronic gastritis were assigned to group C (chronic).

Assessment of H. pylori status

Two biopsy specimens, one from the antrum and one from the gastric body, were taken routinely for assessment of the state of *H. pylori* infection. The specimens were fixed in 10% formalin, embedded in paraffin, and cut into sections of 4–5 μm. The sections were stained with hematoxylin and eosin (H&E) and with Giemsa. One experienced observer, who was blinded to the results of the other tests, confirmed the presence of chronic gastritis in H&E-stained biopsy specimens and Giemsa-stained sections used to detect *H. pylori*.

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

Specific primers were designed for the *CDX1/2*, mucin markers (*MUC2*, *MUC5AC*), intestinal metaplasia-associated antigenic molecules (*SI*, *HD*, *ALP*), the gene marker for fundic gland area (H^{+}/K^{+}ATPase β subunit) and the gene for entire gastric glands (pepsinogen C). Similarly, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as an internal control.

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 from the tissues was isolated 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) according to the conditions recommended by 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*, 62°C for GAPDH); extension, 30 s at 72°C. These reactions were repeated for 35 cycles (25 cycles for H^{+}/K^{+}ATPase β subunit and pepsinogen C).

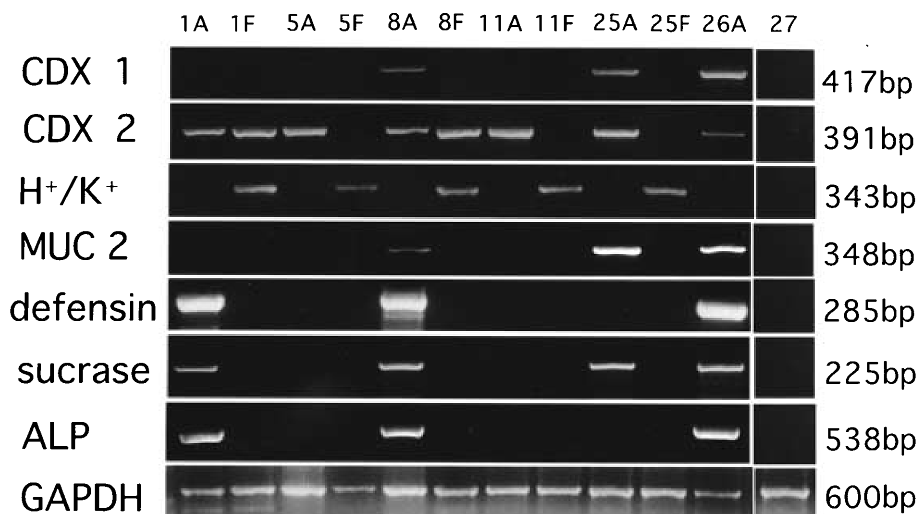

Fig. 1. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *CDX1/2*, the gene marker for fundic gland area (H^+/K^+ATPase β subunit), mucin marker (*MUC2*), and intestinal metaplasia-associated antigenic molecules (*HD*, *SI*, *ALP*). *Left*, genes; *right*, sizes of the PCR products. *Lane numbers* correspond to Table 2 numbers. *A*, Antral mucosa; *F*, fundic mucosa. *Lanes 1*, *5*, *8*, *11*, *25*, *26*, *Helicobacter pylori*-positive cases. *Lane 27*, *Helicobacter pylori*-negative case. The results are summarized in Tables 2 and 3

The PCR products were electrophoresed through a 2.0% agarose gel and stained with ethidium bromide. We confirmed the nucleotide sequences of the RT-PCR products by sequencing (data not shown). The value of the signal intensity ratio between each target gene and GAPDH was analyzed. Image analysis was performed with Gel-Pro Analyzer, Version 2.0 (Media Cybernetics, Silver Spring, MD, USA).

Features of intestinal metaplasia were judged to be present when there was expression of more than one of the intestinal metaplasia-associated genes (*HD*, *ALP*, and *MUC2*) in addition to *SI* mRNA being detected.

HD and *ALP* were utilized as markers of complete type intestinal metaplasia. When the expression of both HD and ALP was negative, the mucosa was defined as incomplete type.

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. Computed two-tailed *P* values of less than 0.05 were regarded as indicating statistical significance.

Results

Clinicopathological findings

Clinicopathological findings of the subjects are summarized in Table 2. Five patients who showed normal mucosa endoscopically and histologically were assigned to group N (specimens 40–44; mean age of the 5 patients, 45.4 years; men/women 3:2).

All patients in group N were negative for *H. pylori* infection. Twenty-six patients who showed chronic gastritis histologically were assigned to group C (specimens 1–39; mean age of the 26 patients, 59.8 years; men/women 15:11). All patients in group C were positive for *H. pylori* infection.

RT-PCR Analysis

All the results of RT-PCR are listed in Tables 2 and 3 and Fig. 1. *MUC 5AC* and pepsinogen C were positive in all specimens, and H^+/K^+ATPase β subunit gene expression was positive in all biopsy specimens from site F.

None of the intestinal gene markers were expressed in group N subjects. Neither *CDX1* nor *CDX2* was detectable (0/5) in the gastric mucosa of group N patients.

Fifty-eight percent (14/24) of the subjects in group C were evaluated as having intestinal metaplasia in the antrum. None of the intestinal gene markers were expressed in the fundic mucosa.

In regard to the type of intestinal metaplasia, two specimens (patient 21, site A; patient 25, site A) were classified as incomplete type. These two antral mucosa specimens expressed *CDX1/2*. Except for these specimens, all specimens were categorized as complete type.

The prevalence of *CDX1* mRNA expression in the antrum was significantly higher in the mucosa with intestinal metaplasia than in the mucosa without intestinal metaplasia (86% (12/14) vs 0% (0/10), $P = 0.0001$) (Fig. 2).

The coexpression of *CDX1* and *CDX2* was observed in 86% (12/14) of intestinal metaplasia samples in the antrum (Fig. 2).

It is of note that the expression of *CDX2* emerged at the stage of chronic gastritis in both antral and fundic

Table 2. Summary of gene expression in 44 samples

			PT-PCR						
Patient no.	Age (years)/sex	Site	*H⁺/K⁺*	*MUC2*	Defensin	Sucrase	*ALP*	*CDX1*	*CDX2*
Group C (n = 39)									
1	48/M	A	−	−	+	+	+	−	+
		F	+	−	−	−	−	−	+
2	50/F	A	−	−	−	−	−	−	+
3	44/F	A	−	−	−	−	−	−	+
4	45/F	A	−	−	−	−	−	−	+
5	61/M	A	−	−	−	−	−	−	+
		F	+	−	−	−	−	−	−
6	48/F	A	−	−	−	−	−	−	+
		F	+	−	−	−	−	−	−
7	74/F	A	−	+	+	+	+	+	+
8	38/F	A	−	+	+	+	+	+	+
		F	+	−	−	−	−	−	+
9	46/F	A	−	+	+	+	−	+	+
10	77/M	A	−	+	+	+	−	+	+
11	71/M	A	−	−	−	−	−	−	+
		F	+	−	−	−	−	−	−
12	64/F	F	+	−	−	−	−	−	−
13	59/M	A	−	−	−	−	−	−	+
14	67/M	A	−	+	+	+	−	+	+
		F	+	−	−	−	−	−	+
15	71/F	A	−	−	−	−	−	−	−
16	65/F	A	−	−	−	−	−	−	+
		F	+	−	−	−	−	−	−
17	74/F	A	−	−	+	+	−	+	+
18	74/M	A	−	+	+	+	+	+	+
19	58/M	A	−	+	−	+	+	+	+
		F	+	−	−	−	−	−	+
20	74/M	A	−	+	+	+	−	+	+
21	44/M	A	−	+	−	+	−	−	+
		F	+	−	−	−	−	−	−
22	73/M	A	−	−	−	−	−	−	+
		F	+	−	−	−	−	−	+
23	60/M	A	−	+	+	+	−	+	+
		F	+	−	−	−	−	−	−
24	61/M	F	+	−	−	−	−	−	+
25	50/M	A	−	+	−	+	−	+	+
		F	+	−	−	−	−	−	+
26	60/M	A	−	+	+	+	+	+	+
		F	+	−	−	−	−	−	+
Group N (n = 5)									
27	47/F	A	−	−	−	−	−	−	−
28	35/F	A	−	−	−	−	−	−	−
29	49/M	A	−	−	−	−	−	−	−
30	69/M	A	−	−	−	−	−	−	−
31	34/M	A	−	−	−	−	−	−	−

RT-PCR, Reverse transcriptase-PCR; A, biopsy specimens from the antrum; F, biopsy specimens from the fundic gland area; H⁺/K⁺, *H⁺/K⁺ATPase β subunit* gene; sucrase, sucrase-isomaltase; *ALP*, alkaline phosphatase

mucosa without expression of *CDX1* or gene markers for intestinal metaplasia (Figs. 2, 3).

In the patients without intestinal metaplasia (patients 5, 6, 11, and 16), *CDX2* tended to be positive in the antrum but was negative in the fundic gland areas. By contrast, in the patients with intestinal metaplasia, *CDX2* was positive in both antrum and fundic gland areas (patients 1, 8, 14, 19, 25, and 26).

Discussion

The intestine-specific transcription factors *CDX1* and *CDX2* are important in the early differentiation and maintenance of the intestinal epithelial cell during gastrointestinal development.[18]

In intestinal metaplasia, gastric epithelial cells undergo changes that transform them into different

Table 3. Signal intensity ratio between each target gene and GAPDH as in Fig. 1

	1A	1F	5A	5F	8A	8F	11A	11F	25A	25F	26A	27
CDX1					0.04				0.23		1.85	
CDX2	0.72	0.53	0.22		0.13	0.8	0.77		0.65		0.25	
H+/K+		0.34		0.2		0.26		0.26		0.33		
MUC2					0.02				1.07		2.81	
Defensin	4.82				1.1						8.33	
Sucrase	0.64				0.29				0.57		2.35	
ALP	2.14				0.48						3.92	

Image analysis was performed with Gel-Pro Analyzer, Version 2.0 (Media Cybernetics, Silver Spring, MD, USA)

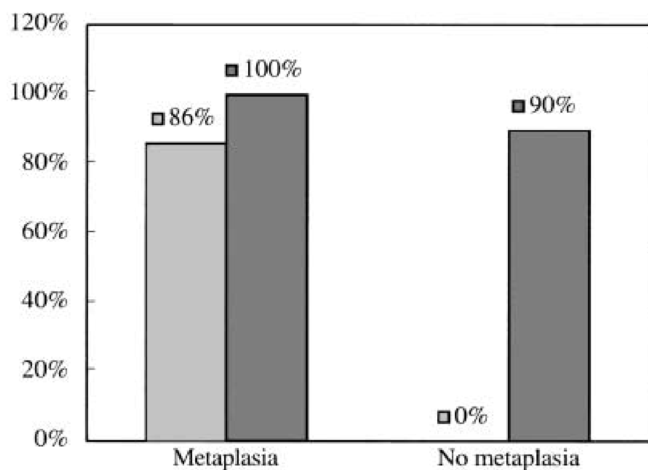

Fig. 2. Expression of *CDX1* (*light gray bars*) and *CDX2* (*dark gray bars*) in the antral mucosa. Expression of *CDX2* emerged in the antrum without the expression of *CDX1* and gene markers for intestinal metaplasia. A high prevalence of *CDX2* mRNA expression was demonstrated in mucosa with or without intestinal metaplasia (100%; 14/14 vs 90%; 9/10). The prevalence of *CDX1* mRNA expression in the antrum was significantly higher in the mucosa with intestinal metaplasia (86%; 12/14) than in the mucosa without intestinal metaplasia (0%; 0/10)

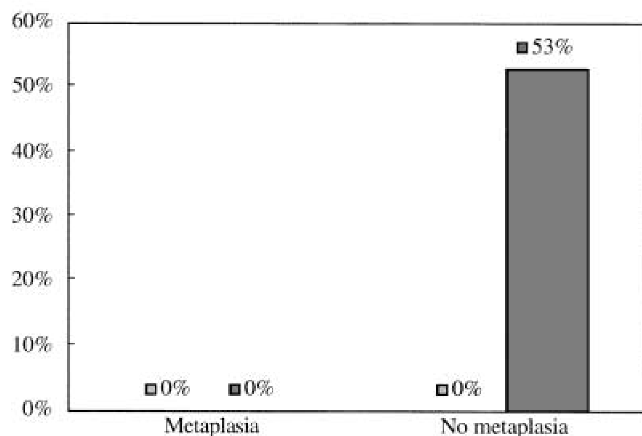

Fig. 3. *CDX1* and *CDX2* expression in the fundic mucosa. Expression of *CDX2* emerged in the fundic mucosa (53%; 8/15) without the expression of *CDX1* and gene markers for intestinal metaplasia. In contrast, expression of *CDX1* was absent in the fundic mucosa (0%; 0/15). *Bars*, As in Fig. 2

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 gastric intestinal metaplasia. It has been proposed that *CDX1* may play an important role in this transdifferentiation.[16] 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*.[16]

In addition to *CDX1*, the homologous transcriptional factor *CDX2* may also participate in this process. Nevertheless, there has been no report to date of the detailed time sequence, i.e., when and how the expressions of these genes is evoked during the process of intestinal metaplasia. This study analyzed the complex patterns of expression of *CDX1* and *CDX2* during the development of intestinal metaplasia.

We studied *CDX1/2* expression in chronic gastritis, intestinal metaplasia, and normal mucosa, and examined whether this expression was correlated with that of gene markers for intestinal metaplasia.

A set of two separate biopsy specimens for RNA extraction and histological examination is not optimal for the detection and analysis of intestinal metaplasia, because intestinal metaplasia is multifocal, and the possibility cannot be denied that sampling errors may occur even if two adjacent biopsy samples are taken side by side.

Consequently, we characterized the specimens based on molecular findings and investigated whether *CDX1/2* expression was correlated with that of gene markers for intestinal metaplasia.

The *CDX1* expression rates appeared to be associated with the transition from chronic gastritis to intestinal metaplasia. We confirmed that *CDX1* expression was more closely associated with intestinal metaplasia than *CDX2*. In contrast to *CDX1*, *CDX2* was already

expressed in chronic gastritis without expression of gene markers for intestinal metaplasia.

In the specimens with *CDX2* expression that were judged as having no intestinal metaplasia, based on our method, intestinal metaplasia-associated genes were not amplified by RT-PCR. This indicates that the mRNA expression level of the genes that confer intestinal phenotype was much lower than that of *CDX2*. Therefore, even if contamination with a small number of metaplastic cells had occurred, it would be difficult to account for the results of our findings in any other way.

Our data also showed that the expression of *CDX2* occurred in the absence of the expression of *CDX1*, *SI*, other intestine-specific genes (*HD*, *ALP*), and *MUC2*. This pattern is consistent with a report that *CDX2* expression in Caco-2 cells induced the expression of *SI* and lactase-phlorizin hydrolase, markers of intestinal differentiation, in vitro.[19]

Both *SI* and lactase-phlorizin hydrolase promoters are activated by Cdx proteins.[3,20,21] 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.[22–25] Our finding implies that the expression of *CDX2* may not be the result of, but, rather, the trigger for, the development of intestinal metaplasia.

Interestingly, the pattern of the relative expression of *CDX1/2* in the development of intestinal metaplasia demonstrated in the present study is similar to that observed during gastrointestinal development in the mouse.[18] Cdx2 protein expression is observed at 9.5 days postcoitum (pc), prior to Cdx1 protein expression, whereas Cdx1 protein expression is mainly seen from 13.5 to 14.5 days pc during the endoderm/epithelial transition.

In our study, *CDX2* expression tended to be positive in the antrum but was negative in the fundic mucosa in stomach lacking features of intestinal metaplasia, whereas *CDX2* tended to be positive in both antral and fundic mucosa in stomach with intestinal metaplasia. Consequently, *CDX2* expression may be evoked in the antrum before expression occurs in the fundic gland mucosa. In the present study, because none of the intestinal gene markers were expressed in the fundic mucosa, it is possible that the extent of chronic gastritis in our patients was not severe. We are currently analyzing *CDX2* expression in various degrees of chronic gastritis by mapping biopsies, using immunohistochemistry. These data will provide insight into abnormal gene expression in the chronic gastritis/metaplasia transition in the stomach.

Atrophic gastritis and gastric intestinal metaplasia are considered to be precancerous pathological conditions that occur during the process of gastric carcinogenesis. Intestinal metaplasia of the human stomach is classified into two types, complete and incomplete. The complete type of intestinal metaplasia was associated with *SI* and *ALP*. Tissue of this type contained goblet cells and Paneth's cells. The incomplete type of intestinal metaplasia is associated with *SI* and goblet cells, but not with *ALP* or Paneth's cells. *HD* and *ALP* were utilized as markers of complete type intestinal metaplasia in this study. Using a definition of incomplete type as being negative for both *HD* and *ALP* expression, we classified two specimens (patient 21, site A; patient 25, site A) as incomplete type, and the remainder as complete. Both types of mucosa expressed *CDX1/2*. Hence, *CDX1/2* expression may be positive regardless of the type of intestinal metaplasia. We are currently analyzing *CDX1/2* expression immunohistochemically to determine the precise localization of their proteins in complete and incomplete type intestinal metaplasia.

Based on the data shown here, it cannot be concluded that *CDX1/2* expression is the sole cause of intestinal metaplasia. However, we have generated a transgenic mouse in which intestinal metaplasia was induced by the expression of *CDX2* in the stomach.[26] Therefore, we consider that *CDX2* expression may play a critical role in the development of intestinal metaplasia.

Furthermore, we are currently analyzing a *CDX1*-expressing transgenic mouse, which also exhibits the intestinal phenotype in the stomach (unpublished data).

To date, the precise molecular mechanisms by which *CDX1* and *CDX2* expression is induced have not yet been elucidated. At present, we are studying the DNA methylation status of the *CDX1/2* promoter region and candidate transcription factors. In our preliminary findings, we detected DNA methylation of the *CDX1* promoter region in gastric tissues (unpublished data). One of the mechanisms of the aberrant *CDX1/2* expression may be the altered methylation status of the promoter region.

In conclusion, the expression of *CDX2* precedes that of *CDX1*, *SI*, other intestine-specific genes (*HD*, *ALP*), and *MUC2* during the progression of intestinal metaplasia.

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