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BRIEF ARTICLE

Expression and significance of homeodomain protein Cdx2 in gastric carcinoma and precancerous lesions

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Abstract

AIM: To investigate the expression and significance of caudal-related homeobox transcription factor (Cdx2) in gastric carcinoma (GC) and precancerous lesions.

METHODS: The expression of Cdx2 in GC, precancerous lesions and normal gastric mucosa were detected using immunohistochemical method. Hematoxylin and eosin staining, alcian blue/periodic acid-schiff and high iron diamine/alcian blue staining were used to classify intestinal metaplasia (IM) and GC.

RESULTS: Cdx2 was not detected in normal gastric mucosa. Cdx2 expression was detected in 87.1% (101/116) of IM, 50% (36/72) of dysplasia and 48.2% (41/85) of GC. The Cdx2-expressing cells in IM were more prevalent than in dysplasia and carcinoma (P < 0.05). There was no relationship between Cdx2 expression and the classification of IM or the degree of dysplasia. Expression of Cdx2 was significantly higher in intestinal-type carcinoma than in diffuse and mixed-type carcinoma (P < 0.05). Positive expression of Cdx2 was mainly found in moderately to well differentiated GC. There was a negative association between nuclear Cdx2 expression and lymph node metastasis and tumor, nodes, metastasis stage of GC (P < 0.05). The patients with Cdx2-positive expression showed a higher survival rate than those with Cdx2-negative expression (P = 0.038). Multivariate analysis revealed that the expression of Cdx2 and lymph node metastasis were independent prognostic indicators of GC (P < 0.05).

CONCLUSION: Cdx2 may be closely related to IM and the intestinal-type GC and implicate better biological behavior and outcome. Cdx2 is useful for predicting the prognosis of GC.

Key words: Caudal-related homeobox transcription factor; Stomach neoplasm; Intestinal metaplasia; Dysplasia; Immunohistochemistry

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INTRODUCTION

Gastric carcinoma (GC) is one of the most common malignant diseases and is the second most common cause of cancer-related death in China and in the world[1]. The pathogenesis of GC is still not very clear. Despite wide acceptance of the gastritis-metaplasia-dysplasia-carcinoma sequence, the precise molecular alterations underlying this progression pathway remain to be delineated[2].

Caudal-related homeobox transcription factor (Cdx2), which is a member of the caudal-related homeobox gene family, plays an important role in mammalian early intestinal development and the maintenance of intestinal epithelia through its regulation of intestine-specific gene transcription[3,4]. Normally, Cdx2 is expressed in small intestinal and colonic epithelia, but not in gastric epithelium[5]. Although Cdx2 expression is restricted to the intestinal epithelium under normal conditions, an ectopic expression has been reported in the gastric mucosa, both in intestine-like metaplasia and in a subset of GCs[6,7]. Recently, Mutoh *et al*[8] and Almeida *et al*[4] reported that the ectopic expression of Cdx2 in the gastric mucosa of transgenic mice is related to the transdifferentiation of gastric mucosal glands into intestinal-like mucosa, and claimed that *Cdx2* gene causes intestinalization in the gastric mucosa. In humans, Cdx2 has been reported to be associated with intestinal metaplasia (IM) in the stomach[6], in which ectopic expression of Cdx2 is speculated to cause the gastric epithelial cells to transdifferentiate into the intestinal phenotype. Several reports have also suggested a tumor suppressor role for Cdx2 in human colorectal carcinogenesis[9-11], and this might also be true for gastric cancers. But the question as to whether the ectopic expression of Cdx2 has any influence on cancer initiation and/or progression in the stomach remains unanswered.

GC is a markedly heterogeneous disease in histologic feature and biological characters, especially in the advanced stages. The clinical evidence showed that the biological behavior and prognosis could be significantly different among the patients with the same stage, histological type, or differentiation grade. Therefore, searching for the biomarkers to indicate the biological characters, and predicting the outcome of patients with GC, is the major focus of research on GC. A number of biomarkers have been found to be involved in the development and progression of GC. Although expression of Cdx2 has been detected in some GCs, few studies reported the relationship between Cdx2 expression and prognosis of GC[12,13].

To better understand the mechanisms underlying malignant transformation and its relationship with developmental processes, we studied and compared the expression of the intestine-specific homeodomain protein Cdx2 in metaplasia, dysplasia and GCs, and the morphologic appearance. Furthermore, in the present study, we analyzed the association between Cdx2 and Lauren's classification, lymph node metastasis, invasion depth, distant metastasis, vascular invasion, tumor size, as well as tumor, nodes, metastasis (TNM) stages, to evaluate the clinical significance of this marker in the histological classification and the prognosis assessment of GC.

MATERIALS AND METHODS

Patients and tissue samples

The present study consisted of 85 cases with surgically resected gastric specimens and 228 cases with endoscopic biopsies were obtained from the Department of Pathology, the First Affiliated Hospital of Anhui Medical University of China from 2000 to 2005, under a protocol approved by the Institutional Review Board. Slides of GC were reviewed to analyze pathologic parameters, including tumor size, histological grading, depth of invasion, and the presence of nodal metastasis. The 85 patients with GCs (aged 20-87 years, mean 61.75 years; 25 females and 60 males) included 20 early cases and 65 advanced cases. Among them, 10 were classified as well-differentiated adenocarcinoma, 34 as moderately differentiated, and 30 as poorly differentiated adenocarcinoma, and 11 as mucinous cell type. Based on Lauren's classification system, all GCs were categorized into three histological types: intestinal, diffuse, and mixed[14]. Forty-three cases were classified as intestinal, 35 as diffuse and 7 as mixed. TNM staging was assessed according to the system established by the American Joint Committee on Cancer (AJCC, 19 at pTNM stage Ⅰ and Ⅱ, and 66 at pTNM stage Ⅲ and Ⅳ). All patients were followed up until January 2010 for a minimum of 5 years. No patient had received chemotherapy or radiation therapy before surgery. In addition, 228 cases of gastric endoscopic biopsies included 10 cases of normal gastric mucosa, 30 cases of chronic superficial gastritis, 116 cases of gastric IM, and 72 cases of gastric dysplasia (39 cases of mild dysplasia, 20 cases of moderate dysplasia and 13 cases of severe dysplasia). The study was approved by the Research Ethics Committee of Anhui Medical University, China. Informed consent was obtained from all patients. All specimens were handled anonymously according to the ethical and legal requirements.

Histochemistry

The samples were fixed with 10% neutral-buffered formalin and embedded in paraffin. Paraffin-embedded samples were serially sectioned at 4 μm and mounted on slides. IM was classified into complete type and incomplete type, using Alcian Blue (pH 2.5)/Periodic Acid-Schiff staining and Alcian blue/high-iron diamine (AB/HID) staining (Baso Diagnostics Inc, China). After deparaffinization and rehydration, the sections were incubated with Alcian Blue pH 2.5 for 30 min, followed by 0.5% periodic acid for 10 min and Schiff solution for 10 min. The sections were then counterstained with modified Mayer's hematoxylin for 5 min, dehydrated with graded ethanols and mounted with coverslip. In complete IM, only goblet cells were stained blue, while in incomplete IM, both the goblet cells and the inter-mediate columnar cells appeared blue.

Immunohistochemistry

After routine deparaffinization and rehydration of the slides, antigen retrieval was done by incubation in modified citrate buffer at 121 ℃ for 20 min. The sections were treated with 0.03% hydrogen peroxide for 5min to block the endogenous peroxidase activity, followed by incubation with anti-Cdx2 monoclonal antibody (1:100, Biogenex, San Ramon, CA, United States) at 4 ℃ over-

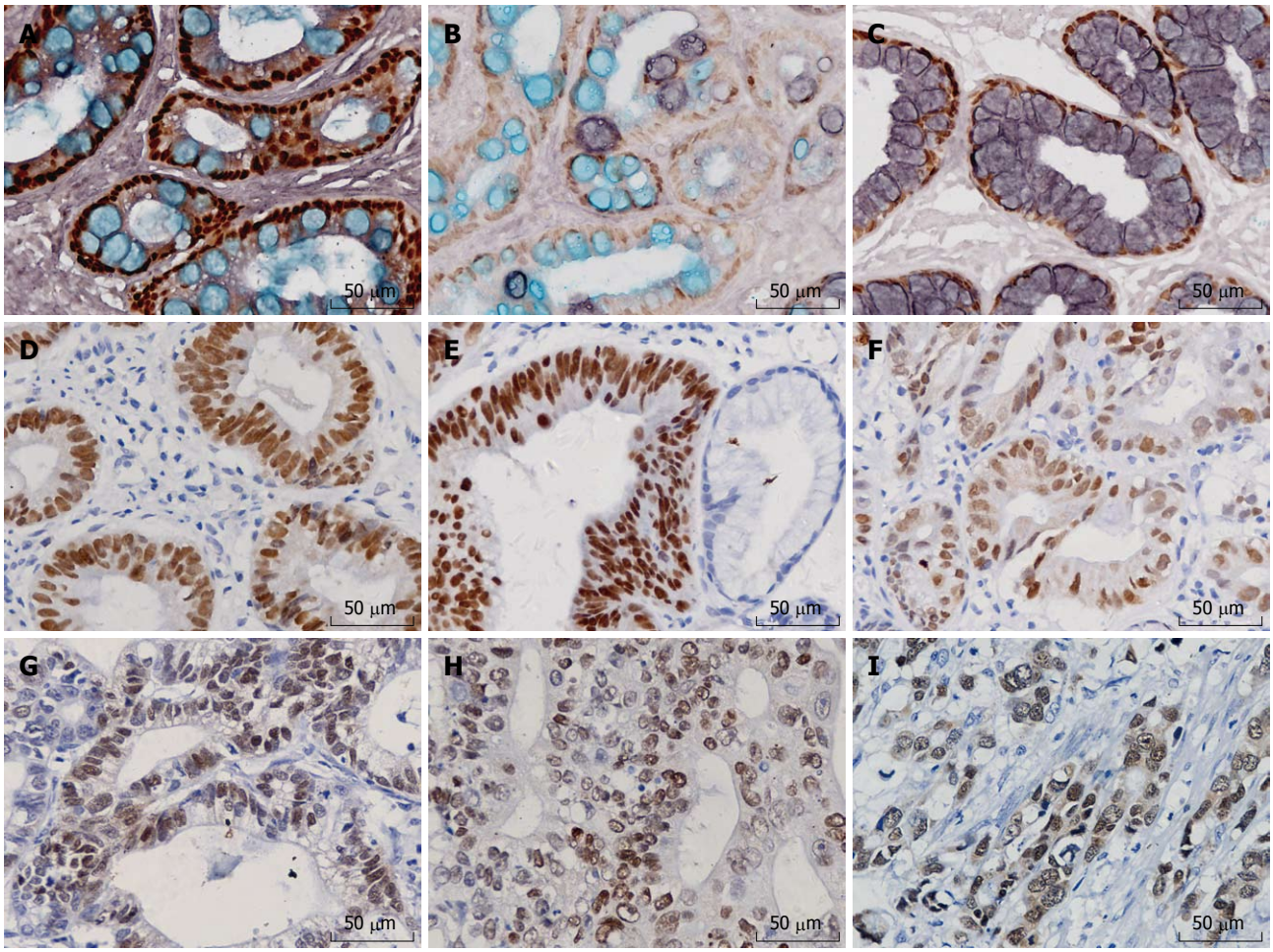

Figure 1 Positive expression of caudal-related homeobox transcription factor in intestinal metaplasia, dysplasia and differentiated gastric cancer. A: Staining of intestinal metaplasia (IM) type Ⅰ (× 400); B: Staining of IM type Ⅱ (× 400); C: Staining of IM type Ⅲ (× 400); D: Staining of mild dysplasia (× 400); E: Staining of moderate dysplasia (× 400); F: Staining of severe dysplasia (× 400); G: Staining of well-differentiated gastric cancer (× 400); H: Staining of moderately-differentiated gastric cancer (× 400); I: Staining of poorly-differentiated gastric cancer (× 400).

night. After washing with 1 × phosphate buffered saline (0.01mol/L, pH 7.4), the sections were subsequently incubated with the biotinylated secondary antibody (Dako, Denmark). The sections were then stained using the Ultra-Sensitive™ Immunohistochemical Staining Kit (Maixin Bio, Fuzhou, China) according to the manufacturer's instructions. After development with 0.05% 3,3'-diaminobenzidine, the sections were counter-stained with hematoxylin, dehydrated with graded ethanols and xylene and then mounted with coverslip. In negative control samples, the primary antibody was omitted and 5% goat serum was used as the primary antibody. Known positive tissue sections served as positive control samples.

Double staining

Double staining of immunohistochemistry and AB/HID staining were applied to detect the Cdx2 expression in different subtypes of IM. Compared to either IHC or AB/HID, combination of both staining was able to detect the protein level and subtypes of IM at the same time.

Assessment of immunostaining in cancer cells

Semiquantitative scores were given as the score of the percentage of positive cells plus the score of the staining intensity. The scoring criteria of the percentage of positive nuclei (Figure 1A-C) were: score 0: 0%-5% positive cancer cells; score 1: 6%-25% positive cancer cells; score 2: 26%-50% positive cancer cells; score 3: 51%-75% positive cancer cells; and score 4: 76%-100% positive cancer cells. The intensity was scored as follows: score 0, no staining; score 1, mild staining; score 2, moderate staining; and score 3, strong staining. The final scores were from 0 to 7. Specimens with a Cdx2 score of 0-2 were considered negative, whereas specimens with a score of 3-7 were considered positive for Cdx2 expression.

Two experienced investigators independently examined the staining who blinded to the clinicopathological data. Different scores between the two investigators were observed in 15% of the cases, and a consensus was achieved in all the cases after discussion.

Statistical analysis

All data were analyzed using SPSS 13.0 software. Fisher's exact test or χ^2 test was used to calculate the association of Cdx2 expression with various clinicopathological features. Cumulative survival was estimated by the Kaplan-

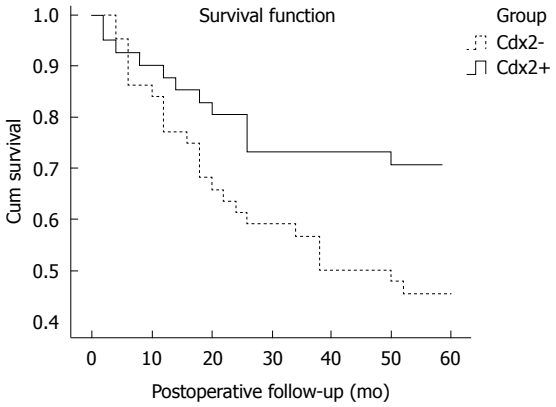

Figure 2 Survival curve of gastric cancer patients with caudal-related homeobox transcription factor positive-expression compared with negative-expression group (*P* = 0.038). Cdx2: Caudal-related homeobox transcription factor.

Meier method and differences between survival curves were analyzed by the log-rank test. The influence of each variable on survival was analyzed by the multivariate Logistic regression analysis. Differences were assumed to be statistically significant when $P < 0.05$.

RESULTS

Cdx2 expression patterns in GC and precancerous lesions

Cdx2 protein expressed in the nuclei of goblet cells of intestinal mucosa, some columnar epithelial cells and some gastric cancer cells. Representative pictures of immunohistochemical staining of Cdx2 are shown in Figure 1.

Expression of Cdx2 protein in GC and precancerous lesions

Gastric IM has been classified as complete (small intestine) or incomplete (colonic) using histochemical staining techniques and into three types based on the staining pattern of its mucins: I (complete), and II and III (incomplete). Type I is the most common type: paneth cells are present and goblet cells secrete sialomucins. Types II and III are characterized by the presence of columnar cells and goblet cells secreting sialomucins and/or sulfomucins; the columnar cells secrete sialomucins in type II and sulfomucins in type III. Cdx2 protein can not be detected in normal gastric mucosa and chronic superficial gastritis. The expression rates of Cdx2 were 87.1% in IM (101/116, Figure 1A-C), 50% in dysplasia tissues (36/72, Figure 1D-F) and 48.2% (41/85, Figure 1G-I) in gastric cancer, respectively. The positive rates of IM, dysplasia and GC were higher than that of normal mucosa ($P < 0.05$). The expression of Cdx2 in IM was much higher than that in dysplasia and carcinoma ($P < 0.05$). There was no significant difference between dysplasia and carcinoma ($P > 0.05$). The expression rates of Cdx2 were 90%, 85% and 75%, respectively in type I, II and III IM, but the difference was not statistically significant ($P > 0.05$). Cases of gastric epithelial dysplasia were graded as mild, moderate and severe dysplasia according to the previously established criteria that included the degree of architectural complexity and cytological atypia[15]. The positive rates of Cdx2 were 43.5%, 55% and 61.5% in mild, moderate and severe dysplasia, showing an increasing trend. But there was no statistically significant correlation between Cdx2 expression and grade of dysplasia ($P > 0.05$, Table 1).

Table 1 Expression of caudal-related homeobox transcription factor in gastric carcinoma and precancerous tissues

Type	Cases	Cdx2 positive cases	%	χ^2	*P* value
Different subtypes of intestinal metaplasia	116	101	87.1	33.826	0.000
IM (type I)	80	72	90	31.400	0.000
IM (type II)	20	17	85	7.426	0.003
IM (type III)	16	12	75	5.365	0.012
Dysplasia	72	36	50	0.004	0.873
Mild	39	17	43.5	0.083	0.700
Moderate	20	11	55	0.088	0.637
Severe	13	8	61.5	0.355	0.553
Gastric carcinoma	85	41	48.2		

Cdx2: Caudal-related homeobox transcription factor; IM: Intestinal metaplasia.

Correlation of Cdx2 expression with clinicopathological parameters in gastric cancer patients

The correlation of Cdx2 expression with clinicopathological parameters in gastric cancer patients is depicted in Table 2. Cdx2 expression was associated with direct Lauren classification, differentiation, lymph node metastasis and clinical stage. The positive rate of Cdx2 in intestinal gastric cancer (67.4%) was significantly higher than that in diffuse and mixed-type gastric cancer (25.7%, 42.9%, $P = 0.001$). The positive rate of Cdx2 in well and moderately differentiated adenocarcinoma (65.9%) was significantly higher than that in the poorly differentiated and mucinous adenocarcinoma (29.3%, $P = 0.001$). In addition, the positive rate of Cdx2 in the group without lymph node metastasis (63.9%) was significantly higher than that with lymph node metastasis (36.7%, $P = 0.017$). The positive rate of Cdx2 of pTNM stage III and IV (40.9%) was also reduced compared with that of pTNM stage I and II (73.7%, $P = 0.018$). Cdx2 expression was not associated with the remaining clinicopathological parameters evaluated, including depth of invasion, sex, and age of patients.

Prognostic implication of Cdx2 nuclear expression in GC

The association between the 5-year survival rate and the expression of Cdx2 was analyzed using the Kaplane Meier method. The results are shown in Figure 2. The patients with nuclear Cdx2 expression showed a significantly higher 5-year survival rate than patients without Cdx2 expression ($P = 0.038$). Based on the Cox regression analysis in the 85 patients, Cdx2 expression and lymph node metastasis seemed to be independent prognostic indicators ($P = 0.001$, $P = 0.019$, respectively, Table 3).

Table 2 Relationship between expression of caudal-related homeobox transcription factor and clinical pathological characteristics of gastric carcinoma

Pathological parameters	*n*	Cdx2 positive cases	Positive rate (%)	χ^2	*P* value
Gender				0.071	0.642
Male	60	30	50		
Female	25	11	44		
Age (yr)				0.000	1.000
⩾ 55	57	28	49.1		
< 55	28	13	46.4		
Lauren classification				13.544	0.001
Intestinal	43	29	67.4		
Diffuse	35	9	25.7		
Mixed type	7	3	42.9		
Differentiation				9.991	0.001
Well and moderately differentiated	44	29	65.9		
Poorly differentiated and mucinous adenocarcinoma	41	12	29.3		
Depth of invasion				5.630	0.131
T1	9	7	77.8		
T2	11	5	45.5		
T3	25	14	56		
T4	40	15	37.5		
Lymph node metastasis				5.089	0.017
No	36	23	63.9		
Yes	49	18	36.7		
Clinical stages				5.102	0.018
Ⅰ + Ⅱ	19	14	73.7		
Ⅲ + Ⅳ	66	27	40.9		

Cdx2: Caudal-related homeobox transcription factor.

Table 3 Association of various factors with overall survival by the logistic regression

Variables	B	SE	Walt	Exp (B)	*P* value
Cdx2	-1.290	0.397	10.586	0.275	0.001
Lymph node metastasis	0.808	0.344	5.501	2.243	0.019

Cdx2: Caudal-related homeobox transcription factor.

DISCUSSION

It was proposed by Correa[16] that human gastric carcinogenesis is a multistep process that progresses from chronic gastritis, atrophy, IM, dysplasia and finally leads to gastric cancer. This model, although challenged by a few, is widely accepted, especially for the intestinal type of gastric cancer. IM is most frequently recognized as a pathological condition in the human stomach, where it often develops from progression of chronic gastritis, and has been extensively studied as a putative preneoplastic lesion of intestinal-type GC. Cdx2 was found to be intensively involved in intestinal metaplastic differentiation[17]. During mouse small intestinal development, Cdx2 is expressed much earlier than Cdx1 in the hindgut[18], and in human IM, expression of Cdx2 precedes that of Cdx1 during the progression of IM, implying that the expression of Cdx2 may trigger the initiation and development of IM[19,20]. Cdx2 may stimulate intestinal proliferation and differentiation by transcriptional activation of intestine-specific proteins (MUC2, sucrase-isomaltase, carbonic anhydrase Ⅰ). Aberrant expression of Cdx2 is prominent in intestinal-type gastric adenocarcinoma and Cdx-2 may therefore play an important role in gastric carcinogenesis, especially in the intestinal type[12]. In this study, we found that Cdx2 was not expressed in normal gastric mucosa and superficial gastritis, but was expressed in 87.1% of IM. The expression of Cdx2 was also higher in intestinal-type than in diffuse-type GC. Cdx2 has been shown to be a key molecule associated with IM and intestinal or differentiated type GC.

IM can be classified into different subtypes by several classification systems. The most widely accepted one is to classify IM into complete type and incomplete type, with the latter carrying a higher risk of gastric cancer. The complete type is characterized by the presence of absorptive cells, paneth cells and goblet cells secreting sialomucins, similar to the small intestinal phenotype. The incomplete type is characterized by the presence of columnar and goblet cells secreting sialomucins and/or sulphomucins, similar to the colonic phenotype. Using alcian-blue/periodic acid Schiff and high-iron diamine-alcian blue technique, Jass *et al*[21] classified IM into three subtypes. Type Ⅰ corresponded to the complete type, while type Ⅱ and type Ⅲ were classified as the incomplete type according to the mucins secreted by the columnar cells: sialomucins in type Ⅱ and sulphomucins in type Ⅲ. Several studies claimed that only type Ⅲ IM is associated with an increased risk of gastric cancer, but other reports cast doubt on it. In this study, staining of both immunohistochemistry and AB/HID was applied to detect the Cdx2 protein level and subtypes of IM at the same time. The expression rates of Cdx2 were 90%, 85% and 75% respectively in type Ⅰ, Ⅱ and Ⅲ IM. There was no difference of Cdx2 expression among type Ⅰ, Ⅱ and Ⅲ IM. But Liu *et al*[22] demonstrated that the expression of Cdx2 was significantly decreased in incomplete IM compared with complete IM. This may be explained by the different antibodies and different staining protocols.

So far, there have been a few reports about Cdx2 expression in gastric epithelial dysplasia. Similar to Woodland's finding[23], we found no relationship between the grade of gastric epithelial dysplasia and Cdx2 expression. But Kim *et al*[24] demonstrated a positive correlation between Cdx2 expression and the increasing grade of dysplasia.

Our data showed that Cdx2 expression was decreased in gastric cancers, when compared with IM and dysplasia. This collective experience may suggest a potential tumor suppressor role for Cdx2, in view of its sequential decrease in expression along with the stepwise gastric carcinogenesis (IM, epithelial dysplasia and gastric cancer). This opinion is shared by Liu *et al*[22] and Xin *et al*[25], who showed that Cdx2 expression is progressively decreased in gastric IM, dysplasia, and cancer. In our study, Cdx2-positive expressing tumors had tendencies towards less invasiveness and fewer lymph node metastases. The Cdx2-

positive patients were found to have a better outcome than Cdx2-negative patients. Multivariate analysis revealed that Cdx2 represents an independent prognostic indicator. The consequences are consistent with the studies by Seno *et al*[12] and Mizoshita *et al*[13]. We can speculate that, while ectopically expressed in gastric cancers, the Cdx2 gene may play a tumor suppressor role in slowing down cancer progression in the stomach, similar to what happens in the colon. Previous studies suggest that Cdx2 is a tumor-suppressor gene with regard to colorectal carcinogenesis. Several reports[26,27] have revealed that Cdx2 could promote differentiation, inhibit proliferation, and increase sensitivity to apoptosis of intestinal epithelial cells, and colon cancer derived cells. Moreover, Cdx2 was documented to up-regulate transcription of p21/WAF1/CIP1[28,29] and PTEN[30], which plays a critical role in differentiation and tumor suppression, and promotes intestinal differentiation as a cyclin-dependent kinase inhibitor, leading to cell-cycle arrest. The role of Cdx2 in gastric cancer still remains unclear and Cdx2 may have different roles depending on cancer type.

In conclusion, our findings indicated that Cdx2 is a special and sensitive marker for IM. We revealed that Cdx2 might be closely related to the intestinal-type GC and implicate better biological behavior and outcome. Cdx2 might be useful in predicting the prognosis of GC. Further studies are necessary to confirm our findings.

COMMENTS

Background

Intestinal metaplasia (IM) of the stomach is a preneoplastic lesion that confers an increased risk for the development of gastric carcinoma (GC), which remains the second leading cause of cancer death worldwide. Caudal-related homeobox transcription factor (Cdx2) is an intestinal transcription factor responsible for regulating the proliferation and differentiation of intestinal epithelial cells. The role of Cdx-2 in GCs is not clearly understood.

Research frontiers

Human Cdx2 is known as a caudal-related homeodomain transcription factor that is expressed in the intestinal epithelium and is important in differentiation and maintenance of the intestinal epithelial cells. Although Cdx2 expression is restricted to the intestinal epithelium under normal conditions, an ectopic expression has been reported in the gastric mucosa, both in intestine-like metaplasia and in a subset of GCs. Although expression of Cdx2 has been detected in some GCs, few studies reported the relationship between Cdx2 expression and prognosis of GC.

Innovations and breakthroughs

This paper evaluated the immunohistochemical expression of the intestine-specific transcription factor Cdx2 in a large number of GC cases, compared with that in gastric IM, gastric dysplasia, superficial gastritis and normal gastric mucosa. The Cdx2-expressing cells in IM were more prevalent than in dysplasia and carcinoma. In GC, expression of Cdx2 was significantly higher in intestinal-type carcinoma than in diffuse and mixed-type carcinoma. Cdx2 might be closely related to IM and the intestinal-type GC and implicate better biological behavior and outcome. Cdx2 might be useful in predicting the prognosis for GC.

Applications

Cdx2 expression in GC may serve as a useful marker for diagnosis, to judge the degree of malignancy and to predict the prognosis of the patients.

Terminology

The Cdx2, located on chromosome 13 in humans and chromosome 5 in mice, is a member of the ParaHox cluster. Cdx2 is from a class of genes encoding transcription factors that constitute mammalian homologues of the Drosophila gene Caudal. Caudal homologues have been identified in a number of organisms including humans and mice and play essential roles in intestinal development and maintenance of the epithelia. In mice and humans, there are 3 caudal homologues: Cdx1, Cdx2 and Cdx4, of which only Cdx2 plays a role in development of the gastrointestinal tract.

Peer review

This is an interesting, well written paper evaluating the immunohistochemical expression of the intestine-specific transcription factor Cdx2 in a large number of GC cases, compared with that in gastric IM, gastric dysplasia, superficial gastritis and normal gastric mucosa. It is the first study indicating a prognostic role of Cdx2 expression in GC.

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