PTEN expression and suppression of proliferation are associated with Cdx2 overexpression in gastric cancer cells

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Abstract. The prognosis of gastric cancer (GC) is associated with Cdx2 and nuclear PTEN coexpression. This study aimed to determine the expression patterns of Cdx2 and PTEN in various GC tissues and cell lines to identify their relationship in GC. Immunohistochemistry was undertaken to assess the expression patterns of Cdx2 and PTEN in paraffin-embedded specimens of 228 GC patients who had undergone radical D2 gastrostomy with long-term follow-up. Cell growth and tumorigenicity were analyzed in the BGC823 cells with exogenous Cdx2 and any changes in the associated signaling pathways were interpreted in exogenous cdx2 expression and cdx2 knockdown. Cdx2 was found in the nuclei of GC cells in 43.4% (99/228) of the paraffin-embedded biopsies. A higher expression of nuclear PTEN was observed in 36.4% (83/228). Coexpression of Cdx2 and nuclear PTEN was detected in GC tumors (59/228, 25.9%) which correlated with the prognosis of advanced GC patients ($p<0.001$). The expression levels of Cdx2 and PTEN were variable in the different GC cell lines. However, the trends were similar between PTEN and Cdx2 in GC tissues and cell lines. High expression of Cdx2 and PTEN significantly reduced tumorigenicity in BGC823 cells compared with the empty vector control. Exogenous expression of Cdx2 triggered the upregulation of PTEN expression and decreased PI3K and pAkt expression and vice versa. The coexpression levels of PTEN and Cdx2 in GC tumors correlated with prognosis in GC patients. Cdx2 may play a role in the upregulation of PTEN by triggering PI3K/Akt inactivation in GC cells.

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Introduction

Gastric cancer (GC) is one of the leading causes of cancer-related death worldwide (1). Although the exact mechanism of gastric carcinogenesis is not fully known, several associated environmental factors have been identified, such as *Helicobacter pylori* infection (2). GC has been classified histologically into either intestinal or diffuse types by the Lauren classification system (3). Intestinal-type cancer is thought to be related to environmental factors and is considered to evolve through well-characterized sequential stages that progress through chronic gastritis, atrophy, intestinal metaplasia, and dysplasia (4). The intestine-specific expression of the homeoprotein, Cdx2, makes it one of the most likely candidates to be involved in inducing intestinal metaplasia of the stomach (5,6).

The *Cdx2* gene encodes for a *Drosophila* caudal-related homeobox transcription factor that regulates the proliferation and differentiation of intestinal cells and maintains the intestinal phenotype (7). While Cdx2 protein is not expressed in the normal stomach; it is highly expressed in the remainder of the normal intestine, in intestinal metaplasia of the stomach, and in a subset of GC (8). Ectopic expression of Cdx2 in the stomach of transgenic mice has shown that Cdx2 is involved in the initiation of intestinal metaplasia formation (9). Mutoh *et al* (10), further observed that intestinal-type GC developed from intestinal metaplasia in *Cdx2*-transgenic mice. Cdx2 has also been adequately validated for the diagnosis of colorectal cancer. The studies that have investigated the relationship between Cdx2 expression and GC prognosis have failed to

result in unanimous agreement (8,11-13). Roessler *et al* failed to observe any significant correlation between levels of Cdx2 and survival probability (14).

Phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) is an important tumor suppression gene and is widely expressed in normal human tissues. Together with the activity of lipid phosphatase and protein phosphatase, it is involved in the regulation of cell growth, proliferation, migration and apoptosis (15). *PTEN* is mutated in a wide range of human cancers and is associated with the apoptosis, proliferation and metastasis of tumor cells (16,17). It is well known that *PTEN* is a tumor suppressor gene that localizes to the cytoplasm and the patients with cytoplasmic expression of PTEN protein have a more favorable prognosis. Recent studies have also reported the involvement of nuclear PTEN involvement in chromosomal stability and GC prognosis (18,19).

A study by Kim *et al* has suggested that Cdx-2 was a target of both PTEN/ phosphatidylinositol 3-kinase (PI3K) signaling and tumor necrosis factor α (TNF-α) signaling via nuclear factor κB-dependent pathways (20). However, the relationship between Cdx2 and PTEN has not been established. Cdx2 protein has an important role in the differentiation of GC, whilst PTEN is closely related to tumor invasion and metastasis. The codetection of these two proteins could provide improved specificity and sensitivity in determining the prognosis of GC (19).

This study aimed to determine the expression patterns of Cdx2 and nuclear PTEN in GC tissue and in different cell lines in order to discover their relationship to clinical and pathological features. The tumorigenic potential of the BGC823 cell line was observed after high expression of Cdx2 and PTEN was established through the exogenous expression of Cdx2. The protein expression of Cdx2, PTEN, PI3K and pAkt was observed after transfecting *Cdx2* into BGC823 cells.

Materials and methods

Patients and follow-up. A group of 228 consecutive patients who presented with GC to Beijing's People's Hospital, Beijing Friendship Hospital and the Beijing Cancer Hospital between 1999 and June 2003 were included in this study. All patients were treated by radical D2 gastrostomy. There were 170 males and 58 females with a mean age of 60.72±12.95 years (range 19-93 years). All patients were followed by clinical evaluation or phone interview until either June 2007 or their death, which provided a minimum of 5 years of follow-up. This study was approved by the Ethics Committee of Peking University People's Hospital, China.

Tissue microarray and immunohistochemistry. Tissue arrays and immunohistochemical analyses were undertaken in accordance with previously published research (19). Briefly, the slides were deparaffinized, and then rehydrated and treated with a 3% hydrogen peroxide solution. After antigen retrieval, the sections were incubated with primary antibodies overnight at 4˚C. Primary antibodies were detected using the Powervision two-step histostaining reagent (Zhongshan, Beijing, China) with PV-6001 and the secondary antibody detection was performed with the use of the diaminobenzidine (DAB) chromogenic reaction. Tissues were counterstained, dehydrated and mounted. Positive and negative controls were included. Two experienced pathologists independently examined the patterns of protein staining in a blinded manner relating to the clinical information. The intensity rating used was: 0, no staining of tumor cells; ±, <10% of cells stained (weak); 1+, 10-50% of cells stained yellow in color (moderate); and 2+, >50% of cells stained deep brown in color (strong). For the purpose of statistical analysis, groups that scored 0, ± and 1+ were combined into a weaker staining group and compared with the group that scored 2+ (strong staining).

RT-PCR. Total RNA from tissue samples and cells were prepared by TRizol reagent (Invitrogen) and cDNA libraries were generated by reverse transcription using Moloney murine leukemia virus reverse transcriptase (MMLV) and oligo(dT) primers. The primers used for *Cdx2* amplification were 5'-AGC CAA GTG AAA ACC AGG AC-3' (forward) and 5'-TTT CCT CTC CTT TGC TCT GC-3' (reverse). The primers used for amplification of the *PTEN* gene were 5'-ACC AGG ACC AGA GGA AAC CT-3' (forward) and 5'-GCT AGC CTC TGG ATT TGA CG-3' (reverse). The β-actin gene was adopted as an internal control for all RT-PCR reactions.

Western blotting. Equal amounts of protein from different samples were electrophoresed on 12% SDS-PAGE and electrotransferred onto polyvinylidene fluoride (PVDF) membranes using Mini PROTEAN 3 system (Bio-Rad). PVDF membranes were blocked with phosphate-buffered saline (PBS) containing 5% fat-free milk powder for 2 h and incubated with primary antibodies (Cdx2, PI3KCA, Abgent; PTEN, pAkt, Santa Cruz; GAPDH, Shanghai Kangchen) at 4˚C overnight. Anti-mouse or anti-rabbit antibodies against IgG conjugated with horseradish peroxidase (HRP) were adopted as the secondary antibodies. Peroxidase activity was visualized with ECL kit (GE Healthcare).

Constructed plasmids. The CDS (coding sequence) region of the human *Cdx2* gene was cloned by polymerase chain reaction (PCR). Sequence analysis was performed to confirm the nucleotide sequences. The following sequences of oligonucleotides were used as primers that contained a linker recognizable by 5'-*Xho*I and 3'-*Eco*RI (underlined): 5'-CGC CTC GAG ATG TAC GTG AGC TAC CTC CT-3' (forward); 5'-TGG AAT TCC TGG GTG ACG GTG GGG TT-3' (reverse). Amplified 942-bp fragments that contained the human *Cdx2* CDS were ligated into the *Xho*I and *Eco*RI sites of pcDNA3.1. According to the previous method, the human *Cdx2* CDS and *PTEN* CDS were ligated into the *Xho*I and *Bam*HI sites of pEGFP-C3 (Clontech).

siRNA-mediated downregulation of gene expression. The target sequence for Cdx2-specific siRNA duplex (Cdx2 siRNA) was derived from an mRNA sequence (5'-AAC CAG GAC GAA AGA CAA AUA-3') of human *Cdx2* and chemically synthesized (Invitrogen) (21). A chemically synthesized mock siRNA (fluorescein-labeled, non-silencing) was also purchased from Invitrogen. Transfection of these

oligos (50 nM) was performed using Lipofectamine 2000 (Invitrogen). For RNA extraction, cells were harvested 48 h after transfection. To measure drug cytotoxicity, cells were grown in 6-well plates then subcultured into 96-well plates 24 h after transfection.

Cell lines and transfection. The GC cell lines BGC823 and SGC7901 were established in the People's Hospital, Peking University, China. AGS and NCI-N87 were purchased from ATCC (American Type Culture Collection). The cells were cultured in complete DMEM (Hyclone) at 37˚C in a humidified atmosphere containing 5% CO_2.

Cells were plated and grown to confluence of 70-90% without antibiotics. Transfections were performed with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. For stable cell expression, cells transfected with *Cdx2*-expressing vectors (pCDNA-*Cdx2*) were selected with 400 μg/ml G418 (Sigma) for 28 days. Clones were picked and expanded for an additional 2 months. Transient transfection was performed in a similar manner, but using PEGFP-*Cdx2* and PEGFP-*PTEN* vectors. Experiments that used transiently transfected cells were undertaken 72 h later. Changes in the size, shape and nuclear morphology of transfected cells were observed with the use of phase-contrast microscopy and compared to cells that were transfected with empty vectors and non-transfected cells.

MTT assay. Monolayer culture growth rate was determined by the conversion of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich China Inc.) to a water-insoluble form. Three thousand cells in 200 μl of medium were plated into 96-well plates and grown under standard conditions. Cultures were assayed on days 0-5. Absorbance values were determined on an enzyme-linked immunosorbent assay reader (ELISA; Bio-Rad) at 570 nm.

Colony formation assays. Five hundred cells were plated into 35-mm plates and allowed to grow for 2 weeks to assess colony formation on the culture plates. Cell colonies were stained with 0.1% crystal violet and the total number of colonies per well was counted.

Flow cytometry. Cells ($5x10^5$) were harvested and washed with PBS and fixed in cold 75% ethanol at 4˚C overnight. After staining with propidium iodide (PI) solution for 30 min, cells were collected on a FACScan flow cytometer equipped with a 488-nm argon laser and analyzed using the CellQuest software (Becton-Dickinson).

Tumorigenic assay in nude mice. To analyze the tumorigenicity of GC cells *in vivo*, $5x10^5$ cells were injected subcutaneously into athymic nude mice. The diameters of the tumors were recorded from the day of tumor cell injection.

Statistical analysis. All data were analyzed using SPSS11.0 software. The association of *Cdx2* and nuclear *PTEN* expression and the associated clinical and pathological features was analyzed using the χ^2 test. P-values of <0.05 were considered to be statistically significant.

Results

Expression of Cdx2 and nuclear PTEN correlated with the clinicopathological features of GC. A favorable prognosis was associated with the following: location in the gastric antrum (p=0.013); intestinal type (p=0.002); well-differentiated (p=0.027); earlier pTNM stage (p=0.001); without lymph node metastasis (p=0.032); and without distant metastasis (p=0.018).

Cdx2 protein was detected in the nuclei of 43.4% (99 out of 228) gastric tumors and nuclear PTEN was highly expressed in 36.4% (83 out of 228). It was found that nuclear PTEN expression is present in normal gastric mucosa, intestinal metaplasia, dysplasia and GC with well-differentiated samples. Cdx2 expression was detected in the nuclei of cells with intestinal metaplasia (33 out of 38), dysplasia (14 out of 17) and well-differentiated GC (45 out of 85), but not in normal gastric mucosa (Fig. 1).

The expression of Cdx2 in the nuclei was significantly higher in stages I and II (59.4%), than in stages III and IV (37.0%, p=0.001). A higher level of expression of Cdx2 was found in intestinal-type cancer (47.6%) compared with diffuse-type GC (19.4%, p=0.002). There was a negative correlation between Cdx2 expression and histological grade (poorly differentiated vs. moderate and well-differentiated tumors; p=0.027). The expression of Cdx2 was found to correlate with tumor location (p=0.013), depth of wall invasion (p=0.002), lymph node metastasis (p=0.032), or distant metastasis (p=0.018, Table I).

A negative correlation existed between high expression of nuclear PTEN and age (p=0.005), differentiation (p=0.022), TNM classification (p=0.003), depth of wall invasion (p=0.017) and distant metastasis (p=0.015). There was a significantly smaller amount of nuclear PTEN expression in the diffuse-type cancer (13.9%) than in the intestinal-type cancer (40.3%, p=0.002). The expression of nuclear PTEN did not correlate with other clinicopathological features (Table I).

Coexpression of Cdx2 and PTEN is correlated with prognosis in GC patients. Tissue biopsies with Cdx2-positive cells had a significantly higher postoperative 5-year survival rate (64.4%) than Cdx2-negative patients (32.5%; p<0.001; Fig. 2A). The nuclear PTEN-positive group had significantly higher 5-year survival rates (56.4%) than the nuclear PTEN-negative group (39.5%; p=0.003; Fig. 2B). Using a Cox regression analysis for the 228 patient samples, Cdx2 nuclear expression (p=0.004), TNM stage (p<0.001), and the Lauren classification score (p=0.001) appear to be independent prognostic indicators (Table II).

Based on the expression profiles of Cdx2 and nuclear PTEN, the 228 patients were categorized into the following: group A, $Cdx2^+$/nuclear $PTEN^+$; group B, $Cdx2^+$/nuclear $PTEN^-$ or $Cdx2^-$/nuclear $PTEN^+$; and group C, Cdx2-/nuclear $PTEN^-$. There were significant differences in the survival rates between group A and one of the other groups (p<0.001; Fig. 2C). The survival rate of group A was significantly higher than that of groups B and C (p=0.001 and p<0.001; Fig. 2C), respectively.

The expression trends of Cdx2 and PTEN were similar following immunoassay analysis in well-differentiated GC,

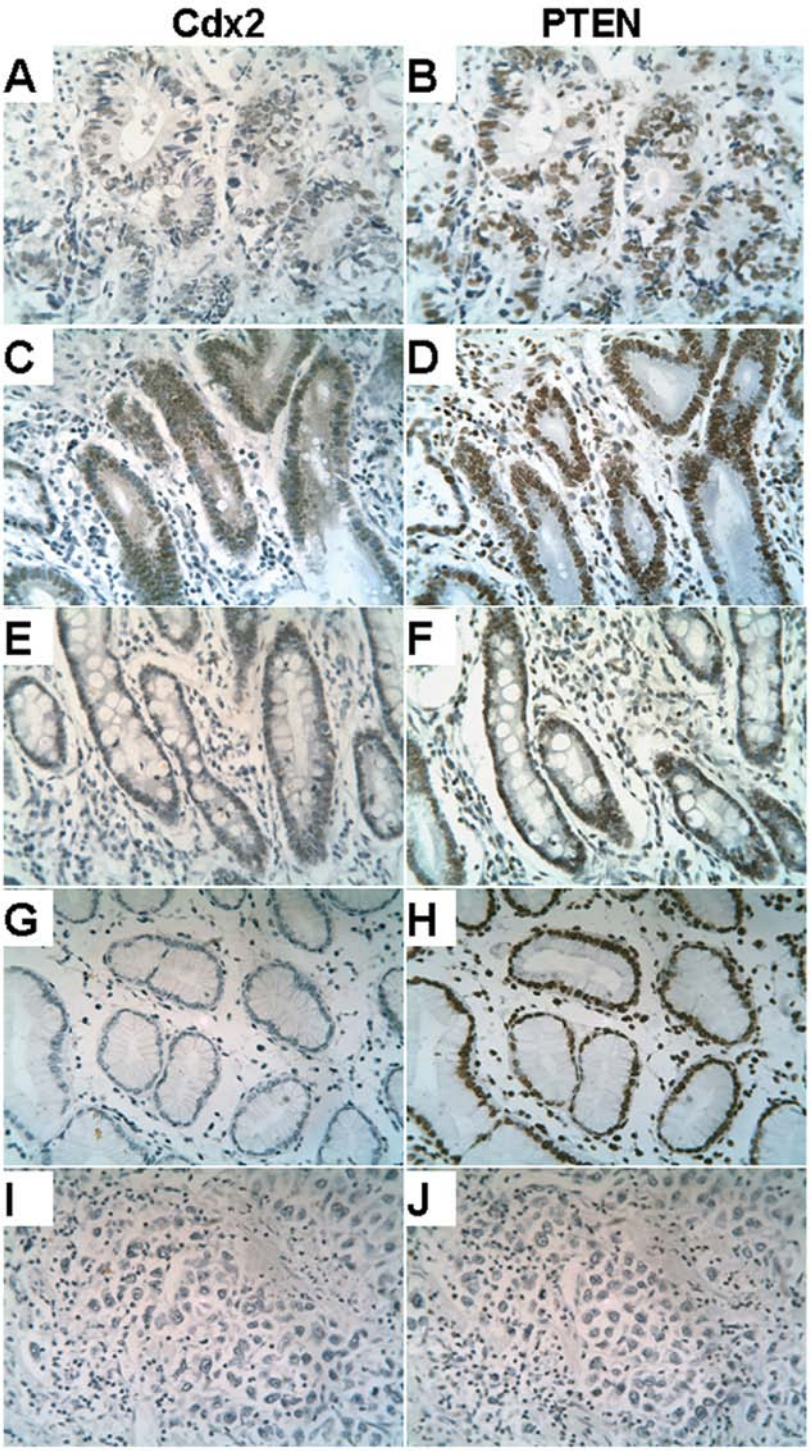

Figure 1. Expression patterns of Cdx2 (A, C, E, G and I) and PTEN (B, D, F, H and J) in gastric cancer and other gastric tissue (brown chromogenic reaction). (A and B) Intestinal-type gastric cancer. (C and D) Dysplasia. (E and F) Intestinal metaplasia. (G and H) Normal gastric mucosa. (I and J) Diffuse-type gastric cancer. Cdx2 and nuclear PTEN have similar expression patterns and highly expressed levels in A, C, E (Cdx2) and B, D, F (PTEN); there is low expression in (I) (Cdx2) and (J) (PTEN). Original magnification, x200.

dysplasia and intestinal metaplasia (Table I). Statistical analysis confirmed a significant correlation and trend between the expression of Cdx2 and PTEN in the group of GC patients (Table III, $p<0.001$). These data suggest that the combined analysis of Cdx2 and nuclear PTEN expression offer significant value in distinguishing between the histological types of GC as well as in assessing the associated prognosis in patients.

Simultaneous expression of Cdx2 and PTEN in different GC cell lines. Expression levels of Cdx2 and PTEN were examined and compared by RT-PCR and western blot analyses in all four GC cell lines. Higher levels of Cdx2 and PTEN mRNA and protein were found in the AGS and N87 cell lines than in BGC823 and SGC7901. Notably, there were low expression levels for Cdx2 and PTEN proteins in BGC823, and higher

Table I. Relationship between Cdx2 or nuclear PTEN expression and clinicopathological features.

		Cdx2 expression			PTEN expression		
	Case	Negative	Positive	P-value	Lower	Higher	P-value
Sex							
Female	58	39	19	0.066	41	17	0.210
Male	170	90	80		104	66	
Age (years)							
19-55	76	46	30	0.479	58	18	0.005
56-90	152	83	69		87	65	
Location							
Corpus or fundus	71	49	22	0.013	48	23	0.371
Antrum	146	74	72		89	57	
Lauren classification							
Intestinal type	191	100	91	0.002	114	77	0.002
Mixed or diffuse type	36	29	7		31	5	
Differentiation							
Poor or undifferentiated	142	89	53	0.027	99	43	0.022
Moderate or well-differentiated	85	40	45		46	39	
TNM classification							
I	30	8	22	0.001	13	17	0.003
II	34	18	16		19	15	
III	119	70	49		76	43	
IV	43	32	11		36	7	
Depth of wall invasion							
T1	16	6	10	0.002	10	6	0.017
T2	26	7	19		10	16	
T3	148	90	58		96	52	
T4	36	25	11		28	8	
Lymph node metastasis							
No	59	26	33	0.032	32	27	0.087
Yes	169	103	66		113	56	
Distant metastasis							
M0	195	104	91	0.018	118	77	0.015
M1	31	24	7		26	5	

Table II. Multivariate analysis of the prognostic factors in 228 cases using the Cox proportional hazard model.

							95.0% CI for exp (B)	
	B	SE	Wald	df	P-value	Exp (B)	Lower	Upper
Lauren classification	-0.882	0.265	11.062	1	0.001	0.414	0.246	0.696
TNM classification			29.260	3	<0.001			
TNM I vs. IV	-2.543	0.626	16.530	1	<0.001	0.079	0.023	0.268
TNM II vs. IV	-1.694	0.395	18.371	1	<0.001	0.184	0.085	0.399
TNM III vs. IV	-0.607	0.248	5.991	1	0.014	0.545	0.335	0.886
Cdx2 expression	0.666	0.229	8.460	1	0.004	1.946	1.243	3.049

B, β regression coefficient; SE, standard error; Wald, test statistics used for the determination of the meaning of variables; df, degrees of freedom; exp (B), exponent; CI, confidence interval. Variables included in the logistic regression model were gender, age, location, Lauren classification, differentiation, TNM stage, invasion depth, lymph node metastasis, distant metastasis and expression of Cdx2 and PTEN.

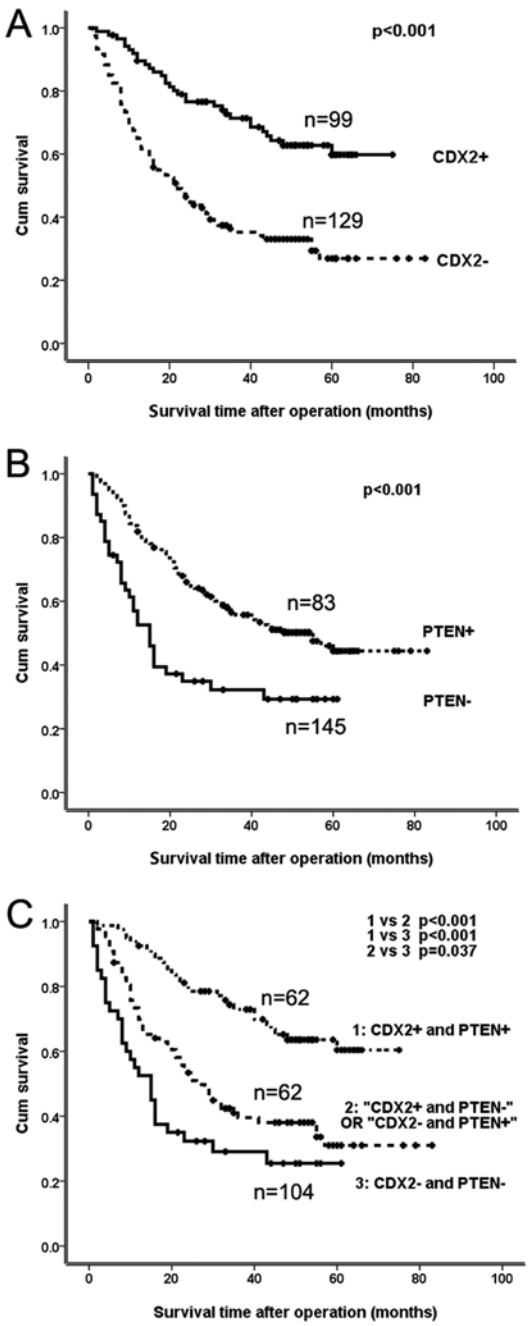

Figure 2. Kaplan-Meier survival curves for 228 gastric cancer patients receiving radical D2 gastrostomy categorized by Cdx2 and/or PTEN nuclear expression. Survival was significantly better for patients with positive Cdx2 or PTEN expression than those with negative expression: (A) p<0.001. (B) p<0.001. (C) Patients with $Cdx2^{+}$/nuclear $PTEN^{+}$ expression profiles had better survival outcomes than others (p<0.001).

levels of expression for PI3K (Fig. 3). The data suggests that there was simultaneous expression of both Cdx2 and PTEN in the different GC cell lines and PI3K were activated in BGC823 cells.

Inactivation of PI3KCA and Akt in BGC823 cells with overexpressed exogenous Cdx2, and vice versa. Transfection of PTEN and Cdx2 genes revealed that Cdx2 and PTEN were overexpressed in Cdx2-BGC823 cells when compared with the control groups. There were no 33-kDa bands in BGC823,

Table III. Comparative analysis of Cdx2 and PTEN expression in GC.

	Nuclear PTEN		
	Weak	Strong	Total
	n (%)	n (%)	228
Cdx2			
Negative	105 (81.4)	24 (18.6)	129
Positive	40 (40.4)	59 (59.6)	99

Pearson correlation, 0.422. p<0.001.

BGC823-EGFP, Cdx2-BGC823 and PTEN-BGC823 cells. A 60-kDa band was seen in Cdx2-BGC823 cells in accordance with the molecular weight of the Cdx2-GFP fusion protein. Compared with the control group, PTEN expression was significantly increased in PTEN-BGC823, but expression changes of Cdx2 did not appear (Fig. 3C and E). GAPDH was used as the internal control. After the transfection of Cdx2 real-time PCR assay demonstrated that the mRNA expression level of both Cdx2 and PTEN increased compared with the control group (Fig. 3D).

It is known that PTEN is a tumor suppressor gene, and that it executes its biological functions through multiple biological mechanisms. Expression of PTEN was obviously induced by Cdx2-BGC823 compared with the control group. The expression of PI3KCA and pAkt was reduced by the transfection of Cdx2 compared with the controls (Fig. 3F). The exogenous expression of Cdx2 induced PTEN expression and PI3K inactivation in BGC823 cells. Similarly, gene knockdown experiments showed the corresponding experimental results (Fig. 3F). After transfection, the exogenous expression of PTEN could not induce Cdx2 expression in the BGC823 cell line, which was inconsistent with previous studies on colorectal cancer cell lines (20).

Increased PTEN expression and suppressed proliferation in BGC823 cells with overexpressed exogenous Cdx2. Cdx2 was transfected into the BGC823 cell line with an efficiency of ~30%. The total quantity of intracellular protein was extracted. A western blot assay was used to observe the increased expression of Cdx2 and PTEN proteins. Consequently, a cell model with high expression of both Cdx2 and PTEN was established (Fig. 3); the BGC823 cell line that was transfected with Cdx2 grew slowly and became larger and more irregular (Fig. 4A).

Since the Cdx2 gene is known to be involved in the regulation of cell differentiation, the overexpression of Cdx2 may result in suppressed cancer cell growth. We found that cells grew significantly slower than the control cells with BGC823-pCDNA (Fig. 4A, B and D). Cell cycle analysis revealed that the population of cells in the G2/M phase was increased by the exogenous expression of Cdx2: 15.89% of Cdx2-BGC823 cells in the G2/M phase of the cell cycle compared with 4.46% of control cells (Fig. 4C); this indicates that Cdx2 and PTEN play an important role in driving cell cycle progression and the promotion of GC cell proliferation.

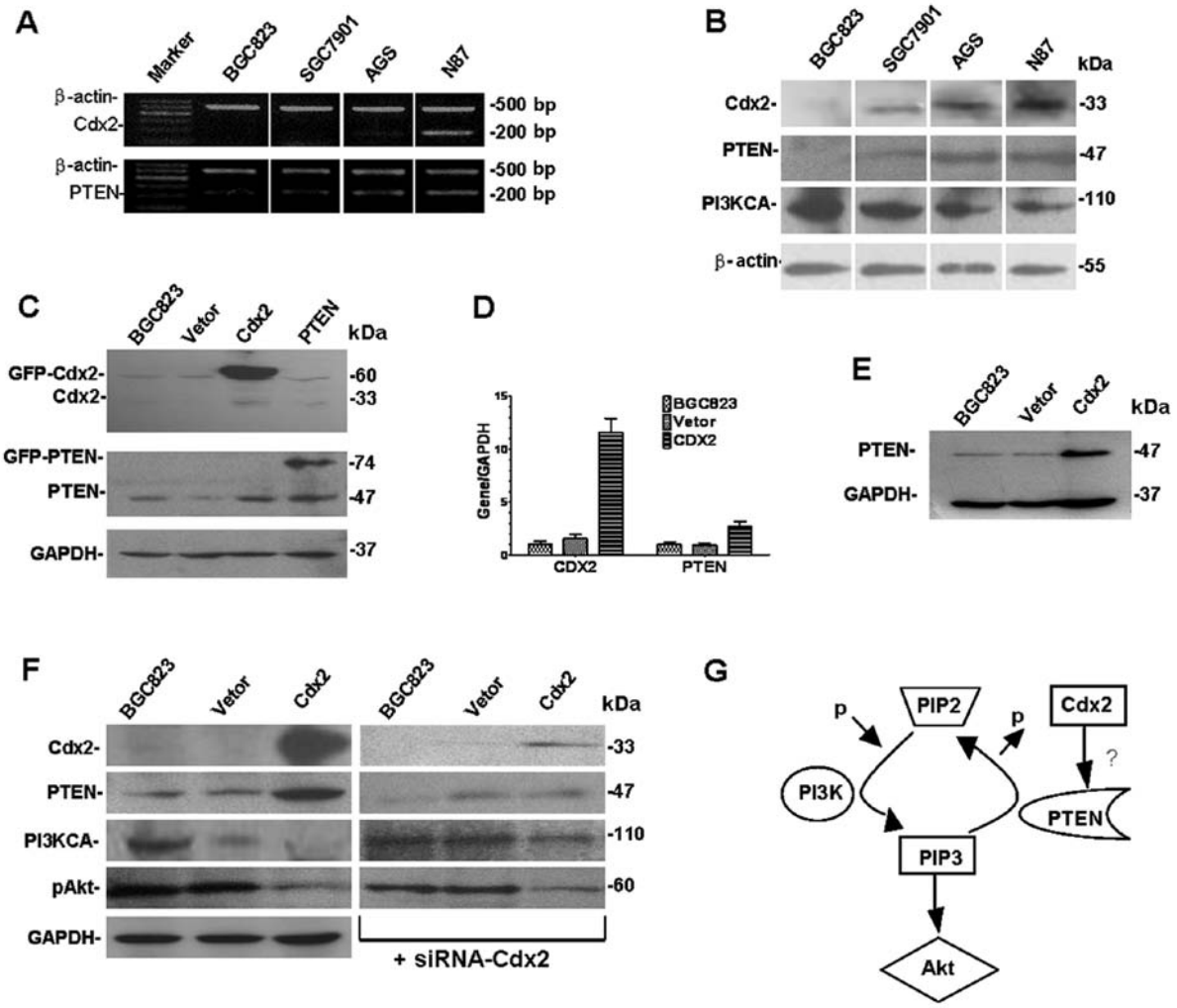

Figure 3. Detection of differential expression of Cdx2 and PTEN in four tumor cell lines by RT-PCR analysis and western blot analysis. (A) *Cdx2* and *PTEN* mRNA. (B) Cdx2 and PTEN protein. The mRNA and protein expression level of both Cdx2 and PTEN were lower or absent in BGC823 than in the other cell lines. This was most prominent in AGS and N87. (C) Endogenous expression of PTEN protein after transfection with a *Cdx2* expression vector or a corresponding empty vector. Endogenous expression of PTEN protein after transfection with *Cdx2* was increased. Endogenous expression of Cdx2 protein after transfection with *PTEN* expression vector or corresponding empty vector was unchanged. Endogenous expression level of Cdx2 protein after transfection with PTEN was unchanged. (D) Expression level of *PTEN* mRNA after transfection with *Cdx2* was increased. (E) Endogenous expression level of PTEN protein after transfection with *Cdx2* was increased. (F) Detection of expressed Cdx2 and PTEN/PI3K/Akt in BGC823 cell lines and confirmation of the presence of the introduced exogenous and knocked-down *Cdx2* gene by western blot analysis. The internal control was GAPDH. The expression levels of PI3K/Akt were reduced after transfection with *Cdx2*. The increased levels of *Cdx2* gene led to increased PTEN expression and decreased PI3K expression. (G) Pathway map.

To confirm the *in vivo* effect on tumors, athymic nude mice were subcutaneously injected with either Cdx2-BGC823 or control cells. The tumors appeared more slowly in the mice injected with Cdx2-BGC823 when compared with the controls (Fig. 4E and F). Furthermore, the average size of the tumors was smaller in the Cdx2-BGC823 mice compared with the controls. These results demonstrate that higher than normal expression of Cdx2 and PTEN led to the inhibition of gastric tumor cell growth *in vivo*.

Discussion

As an intestine-specific transcription factor, Cdx2 has been shown to play a key role in regulating the proliferation and differentiation of intestinal cells and in maintaining intestinal phenotypes (5,22). Cdx2-positive patients in this study had an improved survival rate compared with Cdx2-negative patients. Multivariate analysis further revealed that the level of Cdx2 protein was an independent prognostic indicator. These results are consistent with reports from earlier studies of Cdx2 expression (8,11-13). Levels of Cdx2 expression were high in the tumor cells of well-differentiated cancer biopsies but were decreased in the poorly-differentiated carcinomas. This study found that nuclear PTEN expression had a negative relationship with distant metastasis, suggesting that nuclear PTEN may inhibit the progression of GC. Nuclear PTEN was associated with a good prognosis. However, the full mechanism of nuclear PTEN expression in intestinal metaplasia and GC requires further investigation. Since Cdx2 and nuclear PTEN are both intestinal markers, and because the initial data of the study found the level of Cdx2 protein to be an independent prognostic factor, the significance of Cdx2 and nuclear PTEN coexpression in the prognosis of GC was analyzed. Patients with positive expression of Cdx2 and nuclear PTEN had the

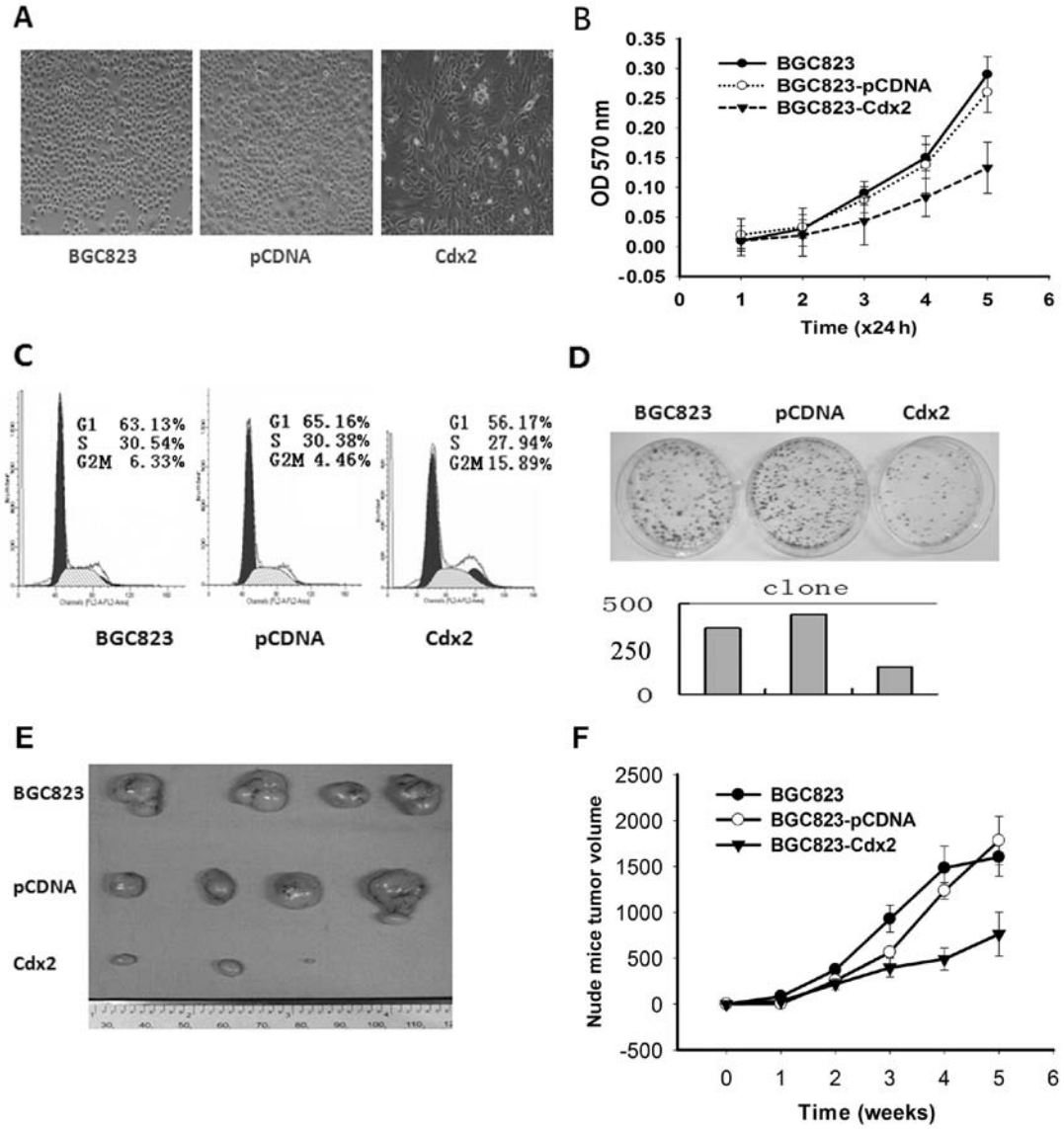

Figure 4. (A) The morphology was significantly altered after transfection with *Cdx2*. Cells that were transfected by an empty vector served as the control. (B) The MTT assay showed that the cell growth of *Cdx2*-BGC823 was significantly inhibited; data are shown as the mean ± SD of three independent experiments ($p<0.01$). (C) FACS analysis confirmed that BGC823-Cdx2 was arrested in the G2/M phase. (D) The colony-forming activities of *Cdx2*-BGC823 decreased significantly; the number of colonies is shown as the mean ± SD of three independent experiments ($p<0.01$). (E) The tumors induced by *Cdx2*-BGC823 was smaller than the controls. (F) Inhibition of tumorigenesis of *Cdx2*-BGC823 was statistically significant compared with the controls ($p<0.01$).

more favorable outcome in this assessment, which suggests that the coexpression of Cdx2 and nuclear PTEN has the potential to be powerful indicator of the prognosis in GC patients.

Cdx2 and nuclear PTEN are both intestinal biomarkers and tumor suppressor genes; their coexpression reduced the degree of malignancy in the GC BGC823 cell line. The present study indicates that the expression patterns of Cdx2 and nuclear PTEN are significantly correlated. Therefore, it was proposed that the expression of Cdx2 and nuclear PTEN may be significantly correlated to GC; the level of nuclear PTEN reflected the potential for metastasis in GC ($p=0.015$) and the level of the intestinal transcription factor, Cdx2, reflected the differentiation of GC ($p=0.002$). Preliminary data found that patients with GC who had high expression levels of both Cdx2 and nuclear PTEN have a lower likelihood of malignancy and better prognosis. A combined analysis of Cdx2 and nuclear PTEN may be useful in predicting the prognosis for GC ($p<0.001$).

Cdx2 promotes the occurrence of intestinal metaplasia and has played an important role in the incidence of GC (5). The level of Cdx2 expression in the AGS and N87 GC cell lines was significantly higher than in BGC823 and SGC7901 in this study. The degree of malignancy of the four GC cell lines (BGC823, SGC7901, AGS and N87) was probably related to the Cdx2 expression level. The BGC823 cell line has a higher malignant potential and more potent tumor-forming potential, but the expression level of Cdx2 is lower in this study (23,24). It was therefore presumed that the malignant potential of the GC cell was negatively correlated to the degree of expression of Cdx2.

There was increased expression of PTEN when Cdx2 was transfected into the BGC823 cell line. As a result, the cell model with a high expression of both Cdx2 and PTEN

was delineated and was similar to that in GC patients with high expression of both Cdx2 and PTEN. In the cell model with a high expression of both Cdx2 and PTEN, there was a significantly slower rate of growth in larger cells, multinucleated cells, and cells that were oval in shape. There was also reduced adhesion, reduced malignant change and capacity for tumorigenicity. *In vitro*, the GC cells that highly expressed Cdx2 and PTEN tended to reduce the degree of malignancy (Fig. 4). This demonstrated that Cdx2 may act as a tumor suppressor gene in the GC BGC823 cell line. However, it has been reported that Cdx2 does not suppress tumorigenicity in the human GC MKN45 cell line (25). Further investigations will be required to clarify the mechanisms that are involved in the regulation of Cdx2 expression.

The Cdx2 gene may be involved in the PI3K/Akt pathway and in the regulation of PTEN expression. PTEN is a tumor suppressor gene with phosphatase activity, in which mutations can often promote tumorigenicity (26). PTEN can dephosphorylate the 3-phosphate of PI (3-5) P3, to reduce the level of phosphorylation Akt-related apoptosis. These findings suggest that the distribution of Cdx2 expression is consistent with that of PTEN in the intestinal mucosa. In human colon cancer cell lines with reduced PTEN expression, the expression of Cdx2 has been found to be significantly decreased. Therefore, PTEN appears to improve the activity of the Cdx2 gene promoter significantly, although methylation of the Cdx2 promoter was not associated with mRNA expression in GC cell lines (27). Inhibiting the activity of PI3K resulted in increased expression of Cdx2. Therefore, Cdx2 was demonstrated to be the target of PTEN/PI3K pathway in a human colon cancer cell line (20). Our studies have found that in BGC823 cell lines that were transfected with pcDNA3.1-Cdx2, expression of PTEN and Cdx2 increased, while PI3KCA and pAkt expression declined. Furthermore, in BGC823 cell lines that were transfected with pcDNA3.1-PTEN, Cdx2 expression was not increased. This suggested that Cdx2 upregulated the expression of PTEN through the PI3K/Akt pathway in the BGC823 cell line. Cdx2 appears to be a downstream target of PTEN regulation in colon cancer cell lines, but this signaling pathway in GC has not been reported to date (20).

There was no Cdx2 expression in normal adult gastric mucosa but there was a high expression of PTEN. It is possible that Cdx2 did not activate PTEN through the PI3K/Akt pathway in normal gastric mucosa, but that PTEN was regulated by a variety of other factors. High expression of Cdx2 in intestinal metaplasia and intestinal-type GC may activate the PTEN/PI3K/Akt pathway but the causal relationship between Cdx2 and PTEN expression is unclear. The regulation of Cdx2 and PTEN through the PI3K/Akt pathway may be via previously unknown branches of PTEN regulation network. The precise mechanism through which the expression of Cdx2 and PTEN proteins are regulated requires ongoing investigation to improve our understanding of the pathogenesis of intestinal type GC.

In conclusion, the combined analysis of Cdx2 and nuclear PTEN expression could enable clinicians to provide a more accurate prognosis in patients with GC. The coexpression of Cdx2 and PTEN may reduce the degree of malignancy in GC cell line BGC823, and Cdx2 might regulate PTEN through the PI3K/Akt pathway.

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