expressed or absent in gastric foveolar epithelium, intestinal epithelium, hepatocytes, and squamous epithelia.[9] A direct association between CK7 expression and poor prognosis of ESCC patients remains unclear. It has been suggested that because CK7 expression is regulated by the forkhead box A1 (FOXA1) transcription factor, FOXA1 may also regulate several cancer-related genes, such as *LOXL2,* in CK7-positive ESCC cases.[8] It has been also reported that CK7 expression is a statistically significant prognostic factor in patients with stage I and II ESCC.[10] However, the impact of immunohistochemically detected CK7 expression on prognosis or therapeutic outcome in patients with stage I–III ESCC remains unclear.

In the present study, immunohistochemical analysis of CK7 was performed in a large number of primary ESCC samples ($n = 225$). In addition, associations between CK7 expression and therapeutic outcomes in stage II and III ESCC patients were investigated.

MATERIALS AND METHODS

Tissue Samples

In a retrospective study design, 225 primary tumors were collected from patients diagnosed with ESCC who underwent surgery between 1990 and 2002 at Hiroshima University Hospital (Hiroshima, Japan). All patients underwent curative resection. All patients underwent right transthoracic esophagectomy with extensive lymph node dissection. Reconstruction was performed with a gastric tube positioned in the posterior mediastinum. Only patients without preoperative radiotherapy or chemotherapy and without clinical evidence of distant metastasis were enrolled in the study. Operative mortality was defined as death within 30 days of patients leaving the hospital, and these patients removed from the analysis. Characteristics of the study population are shown in the Supplementary Table 1. The disease-free survival (DFS) median follow-up time was 25 months (range 1–80 months) and the overall survival (OS) median follow-up time was 27 months (range 1–80 months). Postoperative follow-up was scheduled every 1, 2, or 3 months during the first 2 years after surgery and every 6 months thereafter unless more frequent follow-up was deemed necessary. Chest X-ray, chest computed tomographic scan, and serum chemistries were performed at every follow-up visit. Patients were followed by the patients' physician until their death or the date of the last documented contact.

For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues. Histologic classification was based on the World Health Organization system. Tumor staging was performed according to the tumor, node, metastasis system stage grouping system.[11] As a retrospective study where written informed consent was not obtained, identifying information for all samples was removed before analysis for strict privacy protection. These procedures were in accordance with the ethical guidelines for human genome/gene research enacted by the Japanese government. This study was approved by the Ethical Committee for Human Genome Research of Hiroshima University (Hiroshima, Japan).

Immunohistochemistry

One or two representative tumor blocks, including the tumor center, invading front, and tumor-associated non-neoplastic mucosa, was examined from each patient by immunohistochemistry. In cases of large, late-stage tumors, two different sections were examined to include representative areas of the tumor center as well as of the lateral and deep tumor invasive front. Immunohistochemical analysis was performed with a Dako Envision+ Mouse Peroxidase Detection System (Dako Cytomation, Carpinteria, CA). Antigen retrieval was performed by microwave heating in citrate buffer (pH 6.0) for 30 min. Peroxidase activity was blocked with 3% H_2O_2–methanol for 10 min, and sections were incubated with normal goat serum (Dako Cytomation) for 20 min to block nonspecific antibody binding sites. Sections were incubated with a mouse monoclonal anti-CK7 antibody (1:50, Clone OV-TL 12/30, Dako Cytomation) for 1 hour at room temperature, followed by incubation with Envision+ anti-mouse peroxidase for 1 hour. For color reaction, sections were incubated with the DAB substrate–chromogen solution (Dako Cytomation) for 10 min. Sections were counterstained with 0.1% hematoxylin. Negative controls were created by omission of the primary antibody.

Expression of CK7 was scored in all tumors as positive or negative. When more than 10% of tumor cells were stained, the immunostaining was considered positive for CK7. By using these definitions, two surgical pathologists (N.O. and K.S.), without knowledge of the clinical and pathologic parameters or the patients' outcomes, independently reviewed immunoreactivity in each specimen. Interobserver differences were resolved by consensus review at a double-headed microscope after independent review.

Statistical Methods

Correlations between clinicopathologic parameters and CK7 expression were analyzed by the chi-square test. Kaplan–Meier survival curves were constructed for CK7-positive and CK7-negative patients. Survival rates were compared between CK7-positive and CK7-negative groups. Differences between survival curves of DFS and OS were tested for statistical significance by the log rank

test. Univariate and multivariate Cox regression was used to evaluate the associations between clinical covariates and DFS or OS. SPSS software was used for these analyses (SPSS, Chicago, IL). Hazard ratio (HR) and 95% confidence interval (CI) were estimated from Cox proportional hazard models. For all analyses, age was treated as a categorical variable (65 years or more vs. less than 65 years). For final multivariable Cox regression models, all variables were included that were moderately associated ($P < 0.10$) with DFS or OS by univariate analysis. A P value of less than 0.05 was considered statistically significant.

RESULTS

Immunohistochemical Analysis of CK7 in ESCC

CK7 staining was detected in 55 (24%) of the 225 ESCC specimens investigated. In the nonneoplastic esophageal mucosa adjacent to the tumor, no CK7 staining was observed in squamous epithelial and stromal cells. As reported previously, the esophageal gland was stained by CK7 (Fig. 1a)[12]. Of the 55 ESCC cases in which CK7 staining was detected, 35 cases showed only weak staining of the cytoplasm of tumor cells. In 20 ESCC cases, strong staining was observed in the cytoplasm of tumor cells (Fig. 1b). Expression of CK7 was observed in some cases of squamous cell carcinoma-in-situ (Fig. 1c). When more than 10% of tumor cells were stained, the immunostaining was considered positive for CK7. In total, 20 (9%) of 225 ESCC cases were positive for CK7. We analyzed the relationship between CK7 expression and clinicopathologic characteristics. CK7-positive ESCC cases were more advanced in terms of T classification ($P = 0.0014$, χ^2 test), N classification ($P = 0.0195$, χ^2 test), and tumor stage ($P = 0.0016$, χ^2 test) than CK7-negative ESCC cases (Table 1). Expression of CK7 was not associated with age, sex, or histologic classification.

Relationship Between CK7 Staining and Prognosis of Patients with Stage 0–III ESCC

The association between CK7 expression and DFS or OS was investigated by Kaplan–Meier analysis in all ESCC samples (stage 0–III, $n = 225$). We found that CK7 expression was significantly associated with both poorer DFS and OS ($P = 0.0044$ and $P = 0.0075$, log rank test, respectively; Fig. 2a). Univariate and multivariate Cox proportional hazards analysis was used to further evaluate the association between CK7 expression and DFS (Supplementary Table 2) or OS (Supplementary Table 3). The univariate analysis indicated that expression of CK7 (HR 2.72; 95% CI 1.32–5.59; $P = 0.0066$), tumor stage (HR 13.89; 95% CI 5.03–38.46; $P < 0.0001$), and adjuvant chemotherapy (HR 1.88; 95% CI 1.07–3.32; $P = 0.0293$) were associated with DFS while age, sex, or histologic classification were not. We next performed multivariate model, which included CK7 expression, tumor stage, and adjuvant chemotherapy. However, CK7 expression was not an independent prognostic indicator of DFS (HR 2.01; 95% CI 0.97–4.17; $P = 0.0616$). The univariate analysis

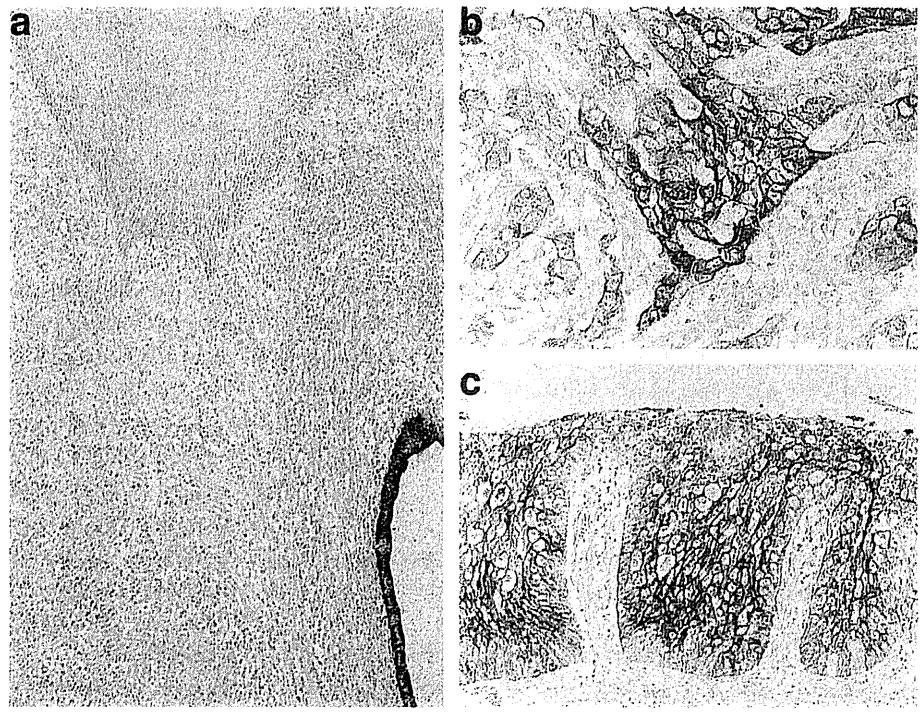

FIG. 1 Immunohistochemical analysis of CK7 in ESCC tissues. In the nonneoplastic esophageal mucosa adjacent to the tumor, esophageal glands were stained by CK7, but squamous epithelium was negative (**a**) (original magnification, 100×). Strong and extensive staining was observed in the cytoplasm of ESCC cells (**b**) (original magnification, 400×). Expression of CK7 was observed in some cases of squamous cell carcinoma-in-situ (**c**) (original magnification, 200×)

TABLE 1 Relationship between cytokeratin 7 (CK7) expression and clinicopathologic characteristics in esophageal squamous cell carcinoma

Characteristic	CK7 expression		
	Positive (%)	Negative	P^a
Age			
<66 years	8 (7)	104	0.4832
≥66 years	12 (11)	101	
Sex			
Male	17 (9)	178	0.7360
Female	3 (10)	27	
T classification			
Tis/1	6 (5)	121	0.0014
T2	1 (5)	21	
T3	12 (16)	62	
T4	1 (50)	1	
N classification			
N0	6 (30)	14	0.0195
N1	120 (59)	85	
Tumor stage			
0/I	4 (4)	97	0.0016
II	5 (8)	60	
III	11 (19)	48	
Histologic classification			
Well/moderate	16 (10)	151	0.7890
Poor	4 (7)	54	

[a] χ^2 test

indicated that expression of CK7 (HR 2.70; 95% CI 1.26–5.78; $P = 0.0110$) and tumor stage (HR 17.24; 95% CI 5.32–55.56; $P < 0.0001$) were associated with OS, while age, sex, and histologic classification were not. Adjuvant chemotherapy was moderately associated with OS (HR 1.81; 95% CI 0.99–3.33; $P = 0.0562$). We also performed multivariate model, which included CK7 expression, tumor stage, and adjuvant chemotherapy. CK7 expression was an independent prognostic indicator of OS (HR 2.24; 95% CI 1.03–4.88; $P = 0.0426$).

We performed survival analysis with other cutoff point. When more than 25% of tumor cells were stained, the immunostaining was considered positive for CK7. In stage 0–III ESCC cases, 9 (4%) of 225 ESCC cases were positive for CK7, and similar results were obtained (data not shown). These results demonstrate that CK7 expression is a useful predictor of OS.

Relationship Between CK7 Staining and Prognosis of Patients with Stage II and III ESCC

Patients with ESCC at stage 0 and stage I have a good rate of survival, whereas patients with ESCC at stage IV show a poor rate of survival. However, it is difficult to predict the survival of patients with stage II or stage III ESCC, and these patients would benefit the most from prognostic biomarkers. Therefore, we analyzed the prognostic value of CK7 expression in patients with stage II and III ESCC ($n = 124$). In Kaplan–Meier analysis, we found that CK7 expression was significantly associated with both poorer DFS and OS (respectively, $P = 0.0472$ and $P = 0.0377$, log rank test; Fig. 2b). Because adjuvant chemotherapy for patients with stage II and stage III ESCC influences survival, we analyzed individuals who did not receive adjuvant chemotherapy ($n = 73$). We found that CK7 expression was significantly associated with both poorer DFS and OS in stage II and III ESCC patients who did not receive adjuvant chemotherapy (respectively, $P = 0.0008$ and $P = 0.0003$, log rank test; Fig. 3a).

To evaluate the potential for CK7 expression as a prognostic predictor in patients with stage II and III ESCC, both univariate and multivariate Cox proportional hazards analyses were used to further evaluate the association of CK7 expression with DFS (Table 2) or OS (Table 3). In univariate analysis, CK7 expression (HR 2.05; 95% CI 0.99–4.27; $P = 0.0548$) was moderately associated with DFS. We next performed multivariate model, which included CK7 expression and the tumor stage. However, CK7 expression was not an independent prognostic classifier of DFS (HR 1.77; 95% CI 0.85–3.70; $P = 0.1297$). In univariate analysis, CK7 expression (HR 2.22; 95% CI 1.02–4.83; $P = 0.0450$) and tumor stage (HR 2.97; 95% CI 1.57–5.62; $P = 0.0008$) were associated with OS. We also performed multivariate model, which included CK7 expression and the tumor stage. CK7 expression was an independent prognostic predictor of OS (HR 2.25; 95% CI 1.01–5.00; $P = 0.0474$).

We performed survival analysis with other cutoff point. When more than 25% of tumor cells were stained, the immunostaining was considered positive for CK7. In stage II and III ESCC cases, 7 (6%) of 124 cases were positive for CK7, and similar results were obtained (data not shown).

Because CK7 staining was detected in ESCC in situ, we analyzed the prognostic value of CK7 expression in patients with stage 0 and stage I ESCC ($n = 101$). When more than 10% of tumor cells were stained, the immunostaining was considered positive for CK7. In total, 4 (4%) of 101 cases were positive for CK7. CK7 expression was not associated with DFS or OS in Kaplan–Meier analysis (data not shown). We also performed survival analysis with another cutoff point. When more than 25% of tumor cells were stained, the immunostaining was considered positive for CK7. In total, 2 (2%) of 101 ESCC cases were positive for CK7. However, CK7 expression was not associated with DFS or OS in Kaplan–Meier analysis (data not shown).

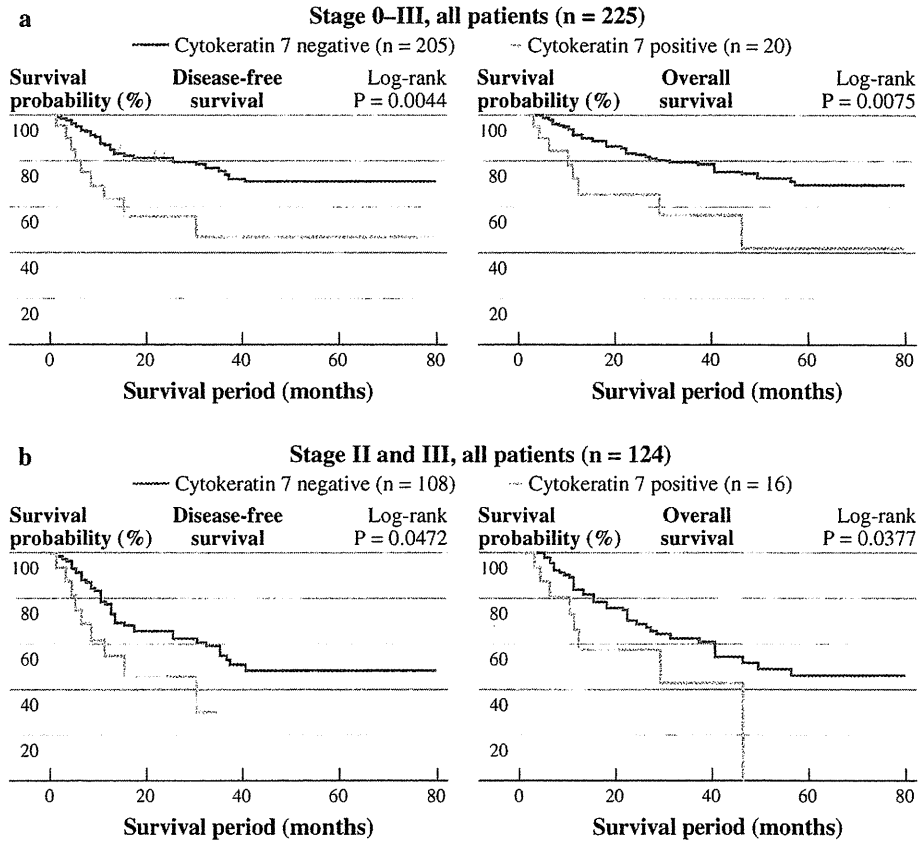

FIG. 2 Disease-free survival and overall survival of patients with ESCC. Kaplan–Meier curves of **a** CK7-positive or CK7-negative ESCC in stage 0–III patients and of **b** patients with stage II and III disease

These results indicate that CK7 expression is associated with OS but not DFS in patients with stage II and III ESCC.

CK7 Expression and Therapeutic Outcome

Biomarkers that can predict therapeutic outcomes may provide tools to allow physicians to better stratify patients to more effective treatments. We analyzed associations between CK7 expression and therapeutic outcomes in stage II and III ESCC patients. Information regarding the administration of adjuvant chemotherapy was available for all patients. Chemotherapy regimens were primarily 5-fluorouracil based. In stage II and III ESCC patients who received adjuvant chemotherapy ($n = 51$), adjuvant chemotherapy was not found to be beneficial. Furthermore, CK7 expression was not significantly associated with therapeutic outcome in patients with stage II and III ESCC (Fig. 3b). These results indicate that CK7 expression cannot predict therapeutic outcome in patients with stage II and III ESCC.

Although we performed survival analysis with another cutoff point, CK7-positive cases were not found in patients who received adjuvant chemotherapy when the cutoff value was set at 25%. Therefore, we set cutoff value at 10% in the following analysis.

Efficacy of Adjuvant Chemotherapy in CK7-Positive and CK7-Negative Patients

We have demonstrated that CK7 expression has the potential to identify patients at high risk of cancer-specific mortality. Furthermore, our data have demonstrated that CK7-positive cases had significantly worse cancer-specific mortality than CK7-negative cases in stage II and III ESCC patients who did not receive adjuvant chemotherapy. These results indicate that CK7 expression is associated with a more aggressive histology of the primary tumor. Because CK7-positive ESCC cases had significantly worse cancer-specific mortality than CK7-negative ESCC cases, adjuvant chemotherapy may be indicated for patients with CK7-positive ESCC. However, it is unclear whether such patients may benefit from adjuvant chemotherapy. To address this issue, we examined whether CK7 expression can identify patients for whom adjuvant chemotherapy is beneficial in stage II and III ESCC. Although in patients with CK7-positive ESCC ($n = 16$) there is a trend toward improved survival with the use of adjuvant chemotherapy in CK7-positive ESCC, therapeutic outcome between patients with CK7-positive and CK7-negative ESCC was not significantly different (Fig. 3c). Conversely, CK7-negative patients with ESCC ($n = 108$) did not benefit

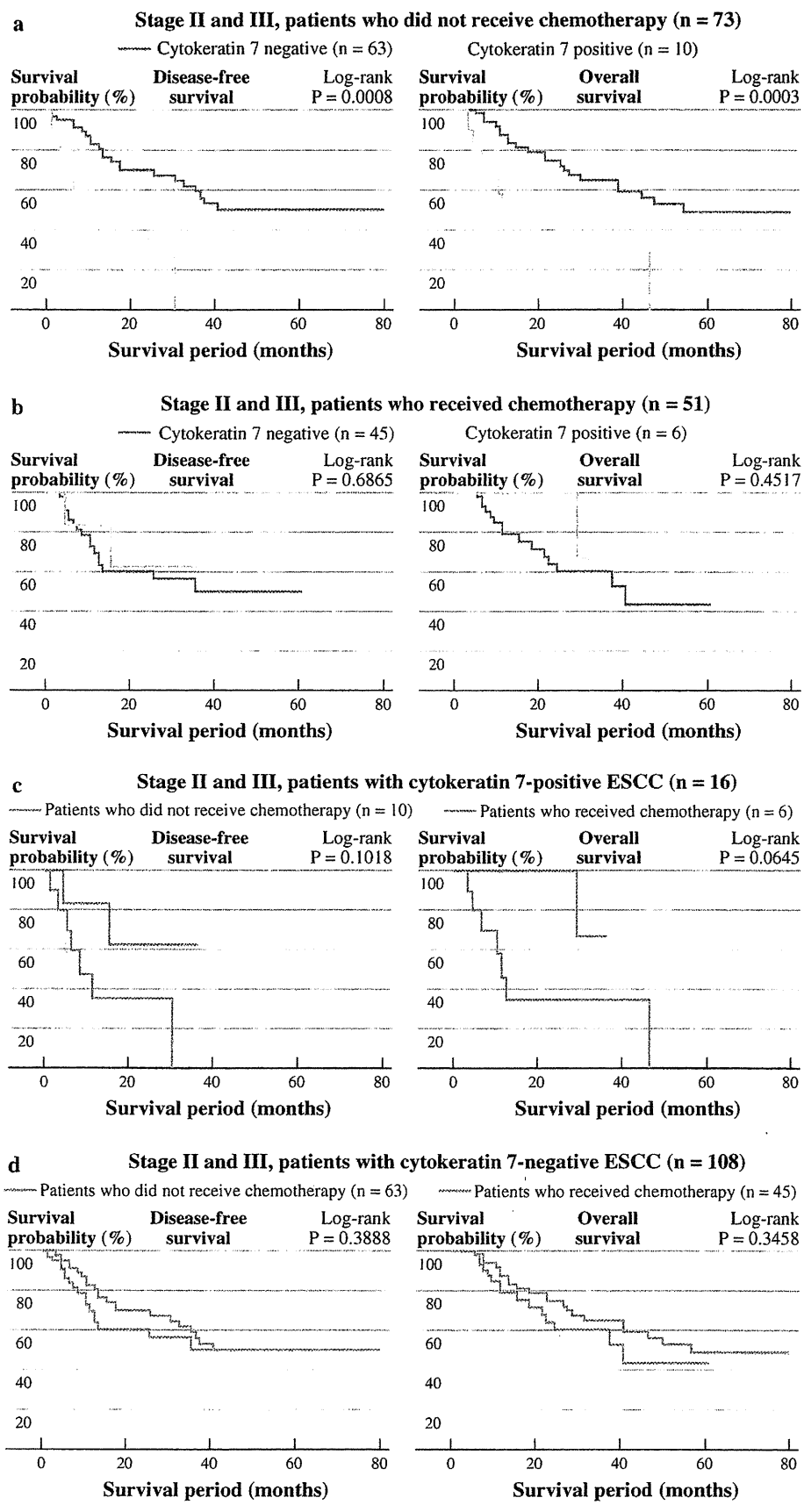

FIG. 3 Disease-free survival and overall survival of patients with stage II and III ESCC. Kaplan–Meier curves of CK7-positive or CK7-negative ESCC in stage II and III ESCC patients who did not receive adjuvant chemotherapy (**a**); stage II and III patients who received adjuvant chemotherapy (**b**); stage II and III CK7-positive ESCC patients with or without adjuvant chemotherapy (**c**); stage II and III CK7-negative ESCC patients with or without adjuvant chemotherapy (**d**)

TABLE 2 Univariate and multivariate Cox regression analysis of CK7 expression and disease-free survival in esophageal squamous cell carcinoma (stage II and III, $n = 124$)

Characteristic	MST, mo	Univariate analysis		Multivariate analysis	
		HR (95% CI)	*P*	HR (95% CI)	*P*
CK7 expression					
Negative	40	1 (Ref.)	0.0548	1 (Ref.)	0.1297
Positive	15	2.05 (0.99–4.27)		1.77 (0.85–3.70)	
Tumor stage					
II	NR	1 (Ref.)	0.0018	1 (Ref.)	0.0032
III	15	2.57 (1.42–4.65)		2.46 (1.35–4.46)	
Adjuvant chemotherapy					
Not received	NR	1 (Ref.)	0.8933		
Received	36	1.04 (0.58–1.86)			
Age					
≥66 years	NR	1 (Ref.)	0.5596		
<66 years	32	1.19 (0.67–2.10)			
Sex					
Female	NR	1 (Ref.)	0.1324		
Male	35	2.46 (0.76–7.94)			
Histologic classification					
Well/moderate	NR	1 (Ref.)	0.3023		
Poor	32	1.37 (0.75–2.50)			

CK7 Cytokeratin 7, *HR* hazard ratio, *CI* confidence interval, *NR* not reached, *MST* median survival time

TABLE 3 Univariate and multivariate Cox regression analysis of CK7 expression and overall survival in esophageal squamous cell carcinoma (stage II and III, $n = 124$)

Characteristic	MST, mo	Univariate analysis		Multivariate analysis	
		HR (95% CI)	*P*	HR (95% CI)	*P*
CK7 expression					
Negative	49	1 (Ref.)	0.0450	1 (Ref.)	0.0474
Positive	29	2.22 (1.02–4.83)		2.25 (1.01–5.00)	
Tumor stage					
II	NR	1 (Ref.)	0.0008	1 (Ref.)	0.0018
III	46	2.97 (1.57–5.62)		2.98 (1.50–5.92)	
Adjuvant chemotherapy					
Not received	46	1 (Ref.)	0.9433		
Received	40	1.02 (0.55–1.91)			
Age					
≥66 years	NR	1 (Ref.)	0.6711		
<66 years	46	1.19 (0.65–2.17)			
Sex					
Female	NR	1 (Ref.)	0.2134		
Male	46	2.11 (0.65–6.80)			
Histologic classification					
Well/moderate	49	1 (Ref.)	0.2689		
Poor	37	1.41 (0.76–2.62)			

CK7 Cytokeratin 7, *HR* hazard ratio, *CI* confidence interval, *NR* not reached, *MST* median survival time

from adjuvant chemotherapy (Fig. 3d). These results demonstrate the potential for the use of CK7 immunohistochemistry to identify patients who can benefit from adjuvant chemotherapy.

DISCUSSION

The long-term survival of patients with ESCC remains poor as a result of the high incidence of lymph node metastasis and early recurrence after curative surgical resection. In the present study, we performed immunohistochemical analysis of CK7 in 225 ESCC cases. Univariate and multivariate analyses revealed that CK7 expression is an independent prognostic classifier of OS in stage 0–III ESCC. We previously reported that patients with CK7-positive ESCC have a worse prognosis for stage I and II ESCC in a cohort from the National Cancer Center Hospital East (Kashiwa, Japan).[10] Therefore, the usefulness of CK7 immunohistochemical analysis is now shown in two independent cohorts. Taken together, these results indicate that immunohistochemical analysis of CK7 is a clinically useful method for prediction of survival of patients with ESCC.

Patients diagnosed with stage II or stage III ESCC have variable prognoses. These groups would benefit from the discovery of prognostic markers that identify individuals for whom adjuvant treatment would be advantageous. In the present study, we demonstrated that CK7 expression was associated with the OS of patients with stage II and stage III ESCC. This indicates that immunohistochemical analysis of CK7 may help identify patients with a high risk of disease recurrence. In the present study, CK7 expression was associated with DFS and OS in stage II and III ESCC patients who did not receive adjuvant chemotherapy. In contrast, CK7 expression was not associated with therapeutic outcome in patients with stage II and III ESCC who received adjuvant chemotherapy. These results suggest that adjuvant chemotherapy may improve survival of patients with CK7-positive ESCC. Although there is a trend toward improved survival with the use of adjuvant chemotherapy in CK7-positive ESCC, therapeutic outcome between patients with CK7-positive and CK7-negative ESCC was not significantly different. As the number of CK7-positive stage II and III ESCC cases was small ($n = 16$), this result should be further investigated in a large ESCC cohort.

The mechanisms underlying the associations between CK7 expression and patients' survival remains unclear. It has been suggested that CK7 expression may be regulated by the FOXA1 transcription factor because *KRT7* mRNA expression levels correlate with *FOXA1* mRNA expression levels.[8] In the TE3 esophageal cancer cell line, knockdown of FOXA1 by siRNA inhibits cell migration and CK7 expression.[8] Therefore, CK7 may be a marker for ESCC expressing FOXA1, and some genes induced by FOXA1 may be associated with cell migration. In addition to CK7, immunohistochemical analysis of FOXA1 may also have the potential to predict patient survival. In the present study, there is a trend toward improved survival with the use of adjuvant chemotherapy in CK7-positive ESCC. To our knowledge, a direct association between CK7 and chemotherapy efficacy has not been investigated. It is possible that some genes induced by FOXA1 may enhance the efficacy of adjuvant chemotherapy in ESCC.

In summary, we have shown that CK7 expression is an independent prognostic classifier in patients with ESCC. Furthermore, there is a trend toward improved survival with the use of adjuvant chemotherapy in CK7-positive ESCC. It is possible that immunohistochemical analysis of CK7 may help identify patients who would benefit from adjuvant chemotherapy. Identification of FOXA1 target genes may improve our understanding of the characteristics of CK7-positive ESCC.

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Reg IV Is a Direct Target of Intestinal Transcriptional Factor CDX2 in Gastric Cancer

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Abstract

REG4, which encodes Reg IV protein, is a member of the calcium-dependent lectin superfamily and potent activator of the epidermal growth factor receptor/Akt/activator protein-1 signaling pathway. Several human cancers overexpress Reg IV, and Reg IV expression is associated with intestinal phenotype differentiation. However, regulation of *REG4* transcription remains unclear. In the present study, we investigated whether CDX2 regulates Reg IV expression in gastric cancer (GC) cells. Expression of Reg IV and CDX2 was analyzed by Western blot and quantitative reverse transcription–polymerase chain reaction in 9 GC cell lines and 2 colon cancer cell lines. The function of the 5′-flanking region of the *REG4* gene was characterized by luciferase assay. In 9 GC cell lines, endogenous Reg IV and CDX2 expression were well correlated. Using an estrogen receptor-regulated form of CDX2, rapid induction of Reg IV expression was observed in HT-29 cells. Reporter gene assays revealed an important role in transcription for consensus CDX2 DNA binding elements in the 5′-flanking region of the *REG4* gene. Chromatin immunoprecipitation assays showed that CDX2 binds directly to the 5′-flanking region of *REG4*. These results indicate that CDX2 protein directly regulates Reg IV expression.

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Introduction

Gastric cancer (GC) is one of the most common human cancers in the world. Cancer develops as a result of multiple genetic and epigenetic alterations [1]. We previously performed serial analysis of gene expression (SAGE) of four primary GCs and identified several GC-specific genes [2]. Of these genes, *regenerating islet-derived family member 4* (*REG4*, which encodes Reg IV protein) is a candidate gene for cancer-specific expression [3]. *REG4* is a member of the *REG* gene family, which belongs to the calcium-dependent lectin superfamily. *REG4* was originally identified by high-throughput sequence analysis of a large inflammatory bowel disease cDNA library [4]. Reg IV is a potent activator of the epidermal growth factor receptor (EGFR)/Akt/activator protein-1 (AP-1) signaling pathway in colon cancer cells and increases expression of Bcl-2, Bcl-xl and survivin, which are proteins associated with the inhibition of apoptosis [5]. Amplification of the *REG4* gene has been reported in pancreatic cancer [6]. Reg IV has been identified as one of the genes up-regulated in cancer-initiating cells [7]. We have previously examined the effect of forced expression of Reg IV in GC cell line. We showed that Reg IV inhibits 5-fluorouracil (5-FU)-induced apoptosis through EGFR activation in GC cells [8]. In contrast, Reg IV-overexpressing cells did not show significant differences in proliferation and invasion activity compared with cells transfected with empty vector [8]. These findings support the notion that Reg IV protein participates in gastric carcinogenesis.

GC can be subdivided into four phenotypes according to mucin expression: gastric or foveolar phenotype; intestinal phenotype; intestinal and gastric mixed phenotype; and neither gastric nor intestinal phenotype [9]. Distinct genetic changes appear to be associated with gastric and intestinal phenotype GC [10]. In our previous observations, Reg IV was expressed in 30% of GC cases and was correlated with intestinal phenotype [11]. A number of immunohistochemical analyses of Reg IV have been reported in human cancers [11–20]. In general, these analyses reported that Reg IV is expressed in adenocarcinoma cells displaying an intestinal phenotype. It has been reported that Reg IV expression is induced by GLI1, which is a key transcriptional factor in the Hedgehog signaling pathway [21], or by growth factors such as EGF, transforming growth factor-α (TGF-α), hepatocyte growth factor (HGF), or basic fibroblast growth factor (bFGF) [22]. However, these molecules are unlikely to account for the association between Reg IV expression and intestinal phenotype differentiation.

We have previously found that expression of Reg IV was correlated with CDX2 expression [11]. CDX2 is a mammalian caudal-related intestinal transcription factor and important for the maintenance of intestinal epithelial cells [23,24]. Several lines of evidence suggest that intestinal metaplasia of the stomach and

intestinal phenotype GC are associated with ectopic CDX2 expression [9,25]. In the present study, we investigated whether CDX2 regulates Reg IV expression in GC and found that CDX2 directly binds to the 5′-flanking region of *REG4* gene and enhances the promoter activity.

Results

Reg IV and CDX2 Expression are Correlated in GC Cells

We first investigated induction of Reg IV expression by CDX2 in GC cell lines. Western blot analysis of CDX2 in 9 GC cell lines revealed that no or low-level expression of CDX2 was detected in MKN-7, TMK-1, HSC-44PE, and KATO-III (**Fig. 1A**). To determine if CDX2 and Reg IV expression were tightly correlated in GC cells, Western blot and quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analyses of Reg IV were performed on 9 GC cell lines. As shown in **Fig. 1A**, Reg IV protein expression was only detected in the 3 cell lines with high levels of *REG4* transcripts measured by qRT-PCR. Of the 5 GC cell lines with CDX2 protein expression, 2 cell lines (MKN-1 and MKN-28) lacked detectable expression of *REG4* transcripts and protein. The cell lines with undetectable CDX2 protein expression (MKN-7, TMK-1, HSC-44PE, and KATO-III) did not show *REG4* transcripts or protein (**Fig. 1A**).

Next, we generated a polyclonal population of MKN-7, TMK-1, HSC-44PE, and KATO-III cells expressing high levels of CDX2 by infection of the cells with replication-defective retroviruses carrying a full-length human CDX2 cDNA because no or low-level expression of CDX2 was detected in these cell lines. However, overexpression of CDX2 failed to activate Reg IV expression by Western blot (**data not shown**). Because it is possible that CDX2 alone is not sufficient for activating Reg IV expression, expression of *CDH17* (encoding LI-cadherin protein), which is one of the targets of CDX2 [24], was also investigated. However, activation of LI-cadherin expression was not found in MKN-7, TMK-1, HSC-44PE, and KATO-III cells expressing high levels of CDX2 (**data not shown**). Because we showed activation of LI-cadherin expression by CDX2 in the HT-29 colon cancer cell line [24], induction of Reg IV expression was investigated in the same cell line. As shown in **Fig. 1B**, induction of Reg IV expression was detected in HT-29 cells infected with retroviruses carrying a full-length human CDX2 cDNA. We also generated a polyclonal population of SW480 (colon cancer cell line) cells expressing high levels of CDX2 by infection of the cells with replication-defective retroviruses carrying a full-length human CDX2 cDNA. As shown in **Fig. 1B**, induction of Reg IV expression was found in SW480 cells infected with retroviruses carrying a full-length human CDX2 cDNA. These results suggest that Reg IV expression can be induced by CDX2 in cell lines derived from colon cancer. Because in intestinal metaplasia of the stomach, CDX2 and Reg IV expression are well correlated [11], the use of a colon cancer cell line might be suitable for the model of intestinal metaplasia.

To better assess the relationship between CDX2 and Reg IV expression, we studied Reg IV expression in an HT-29-derived line with tightly regulated CDX2 activity. We used a polyclonal HT-29 cell line that had been transduced with the pCDX2-ER vector. The pCDX2-ER vector encodes a chimeric protein in which full-length CDX2 sequences are fused upstream of a mutated estrogen receptor (ER) ligand-binding domain. The mutated ER ligand-binding domain no longer binds estrogen, but retains the ability to bind tamoxifen. Treatment of the HT-29/CDX2-ER cell line with 4-hydroxytamoxifen (4-OHT) resulted in strong induction of Reg IV protein expression within 48 hours (**Fig. 1C**). These results indicate that Reg IV is a direct or primary target gene regulated by CDX2. However, CDX2 alone is not sufficient for activating Reg IV expression.

Inhibition of CDX2 by RNA Interference (RNAi) Results in the Down-regulation of Reg IV in GC Cells

To determine whether CDX2 is necessary for Reg IV expression in GC cells, we analyzed the effect of inhibiting CDX2 expression by RNAi in the level of Reg IV expression in HSC-39 cell line because high endogenous CDX2 and Reg IV expression was detected in HSC-39 cell line. CDX2-specific small interfering RNAs (siRNAs) significantly suppressed CDX2 protein expression 3 days after transfection, and expression of Reg IV transcript was down-regulated approximately 50% by CDX2 siRNAs in HSC-39 compared with its levels in control siRNA-treated cells (**Fig. 1D**). These results indicate that CDX2 is involved in maintaining Reg IV gene expression.

Functional Characterization the 5′-flanking Region of *REG4* Gene by Luciferase Assay

To identify potential CDX2-binding sites in the *REG4* promoter region, a search of the genomic sequences immediately 5′ to the presumptive transcription start site was performed, using a consensus binding element for the CdxA chicken *caudal* homologue (5′-A, A/T, T, A/T, A, T, A/G-3′) [26] and a previously described search algorithm [27]. We found four putative CDX2-binding sites in the 2 kilobase (kb) 5′-flanking region of the *REG4* gene (**Fig. 2A**). These were: site A (5′-AATAATA-3′, from −1828 to −1834), site B (5′-CTTTACAG-3′, from −901 to −908), site C (5′-TTTTATGG-3′, from −114 to −121), site D (5′-AATAATA -3′, from −90 to −96). To assess the role of these presumptive CDX2-binding sites in regulating *REG4* transcription, various reporter gene constructs were generated. As shown in **Fig. 2B**, reporter gene constructs containing 2.1, 1.2, or 0.6 kb of 5′-flanking sequence from the *REG4* gene showed strong activity in the HSC-39 cells, which display strong endogenous expression of *REG4* transcripts and protein. By comparison, MKN-1 cells have little endogenous *REG4* transcript and displayed little or no transcriptional activity induced by the 2.1, 1.2, or 0.6 kb *REG4* reporter gene constructs (**data not shown**). The *REG4* reporter gene constructs containing base pairs −116 to +58 and −87 to +58 had reduced activity in the HSC-39 cells (**Fig. 2B**), indicating that sequences between base pairs −634 and −116 play a key role in activating *REG4* transcription. Furthermore, we analyzed single and multiple mutations in the presumptive CDX2-binding sites in the 5′-flanking region of the *REG4* gene using HSC-39 cells (**Fig. 2C**). As expected, presumptive CDX2-binding site C, which is located between base pairs −634 and −116, plays a crucial role in activating *REG4* transcription.

CDX2 Directly Binds to the 5′-flanking Region of *REG4* Gene

To analyze whether CDX2 directly binds to the putative CDX2-binding sites in the *REG4* 5′-flanking region, we performed chromatin immunoprecipitation (ChIP) assays using HSC-39 cells. Using 6 primers for the *REG4* 5′-flanking region (**Fig. 3A**), we recovered DNA fragments containing the *REG4* 5′-flanking region by primer 1, which encompasses presumptive CDX2-binding site C (**Fig. 3B**). DNA fragments from the 5′-flanking region of *REG4*, which were generated using primers 2, 3, 4, 5 and 6 such that they did not contain presumptive CDX2 binding sites, were not recovered by the anti-CDX2 antibody. The specificity of recovery of the *REG4* promoter region following ChIP with anti-CDX2

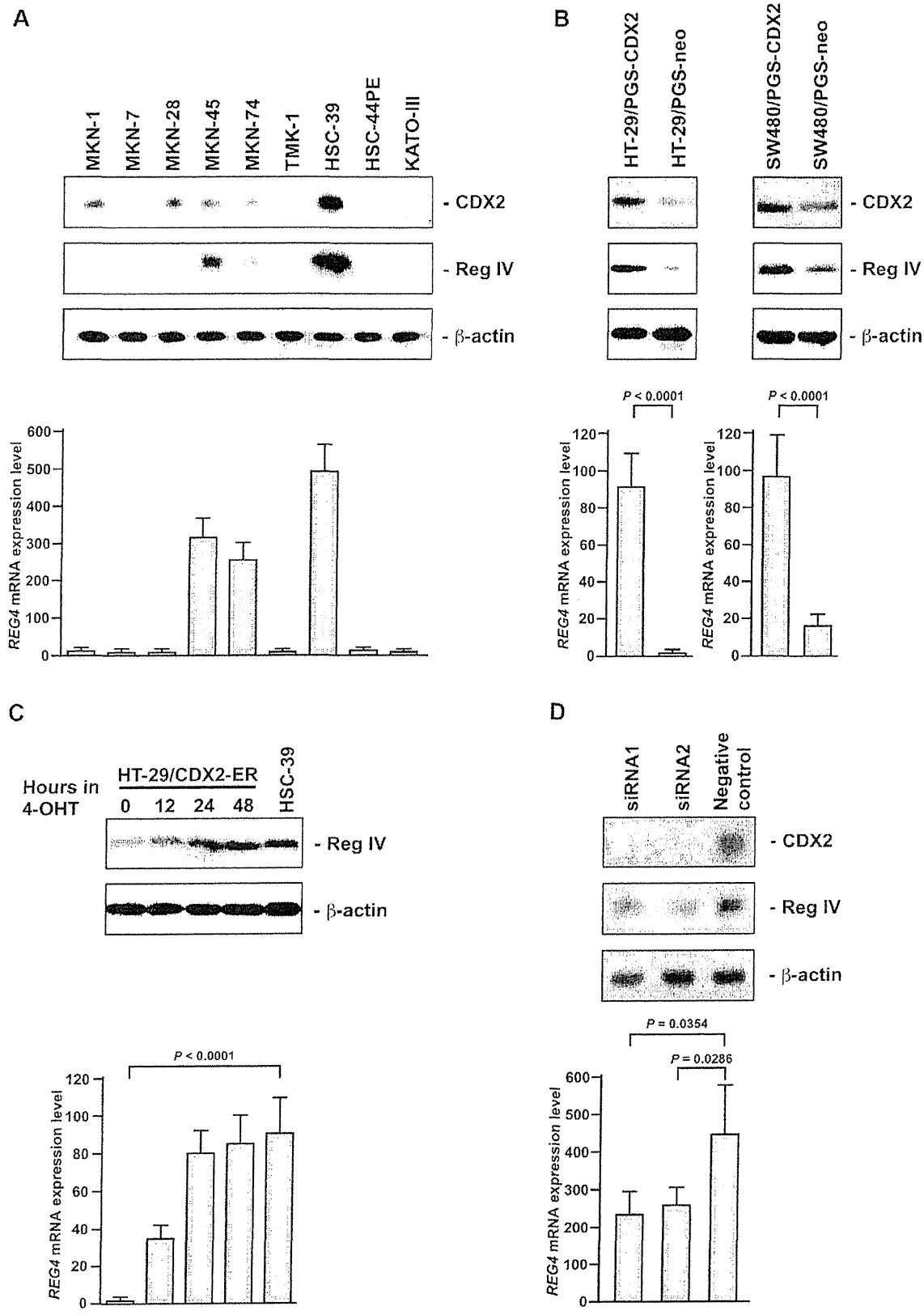
A
MKN-1
MKN-7
MKN-28
MKN-45
MKN-74
TMK-1
HSC-39
HSC-44PE
KATO-III
- CDX2
- Reg IV
- β-actin
REG4 mRNA expression level
B
HT-29/PGS-CDX2
HT-29/PGS-neo
SW480/PGS-CDX2
SW480/PGS-neo
- CDX2
- Reg IV
- β-actin
P < 0.0001
P < 0.0001
REG4 mRNA expression level
C
Hours in 4-OHT
HT-29/CDX2-ER
0 12 24 48
HSC-39
- Reg IV
- β-actin
P < 0.0001
REG4 mRNA expression level
D
siRNA1
siRNA2
Negative control
- CDX2
- Reg IV
- β-actin
P = 0.0354
P = 0.0286
REG4 mRNA expression level

Figure 1. Induction of Reg IV expression by CDX2. A: Western blot analysis of CDX2, Reg IV, and β-actin and qRT-PCR analysis of *REG4* in 9 GC cell lines. B: Western blot analysis of CDX2, Reg IV, and β-actin and qRT-PCR analysis of *REG4* in HT-29/PGS-CDX2, HT-29/PGS-neo, SW480/PGS-CDX2, and SW480/PGS-neo. C: Western blot analysis of Reg IV and β-actin and qRT-PCR analysis of *REG4* in HT-29/CDX2-ER. Time course of *REG4* gene induction in response to activation of a CDX2-ER fusion protein by 4-OHT was analyzed. D: Western blot analysis of CDX2, Reg IV, and β-actin and qRT-PCR analysis of *REG4* in HSC-39 cells transfected with CDX2 siRNA (siRNA1 and siRNA2) and the negative control siRNA. The units of *REG4* mRNA expression level are arbitrary. *P* values were calculated using Student's t-test. * N.S. = not significant.
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antibody was shown by the fact that other irrelevant DNA fragments lacking CDX2-binding sites (e.g., exon 3 of the *CDX1* gene) were not recovered (**Fig. 3B**). In addition, mock immunoprecipitation (mouse IgG) yielded few *REG4* or *CDX1*-specific DNA fragments (**Fig. 3B**). All these findings suggest that CDX2 activates *REG4* transcription by directly binding to sequences in the 5′-flanking region of the gene.

Trimethylation of Histone H3 Lysine 27 (H3K27me3) on the *REG4* Promoter in the GC Cell Lines

Although CDX2 protein expression was found in MKN-1 and MKN-28 cell lines, these 2 cell lines lacked detectable expression of *REG4* transcript and protein. Because it has been reported that DNA hypermethylation of CpG islands is associated with silencing of several genes [28], we investigate whether DNA methylation induced transcriptional inactivation of Reg IV in MKN-1 and MKN-28 cells. We treated these cells with a demethylating agent, 5-aza-2′-deoxycytidine (Aza-dC) and then performed qRT-PCR. However, Reg IV expression was not restored in these cell lines (**data not shown**), suggesting that DNA methylation is not likely to affect Reg IV expression. It has been also reported that H3K27me3 has been associated with repressed gene expression [29]. We further investigated H3K27me3 in GC cell lines. To determine the enrichment of H3K27me3 on the *REG4* promoter in the GC cell lines, ChIP assays were performed. In MKN-1 and MKN-28 cell lines, H3K27me3 levels on the *REG4* promoter region were high, whereas in HSC-39 cell line, H3K27me3 level on the *REG4* promoter region was low (**Fig. 3C**). These results suggest that closed chromatin structure of *REG4* promoter can inhibit Reg IV expression by CDX2.

Discussion

Although it has been reported that Reg IV expression is induced by GLI1 [21] or EGF [22], these molecules are unlikely to account for the association between Reg IV and intestinal differentiation. In the present study, we showed that endogenous CDX2 and Reg IV expression were well correlated in GC cell lines. In addition, using an ER-regulated form of CDX2, we found that there was rapid induction of Reg IV expression after 4-OHT treatment. Reporter gene assays revealed an important role for consensus CDX2 DNA binding elements in the *REG4* promoter region in its transcription. Subsequent ChIP assays showed that CDX2 binds directly to the *REG4* promoter. We previously showed that in primary GC tissue and intestinal metaplasia of the stomach, CDX2 and Reg IV expression were well correlated [11]. These results indicate that CDX2 protein directly regulates Reg IV expression in GC and intestinal metaplasia of the stomach.

CDX2 is overexpressed in intestinal phenotype GC and in intestinal metaplasia of the stomach [9,25]. In contrast, loss of CDX2 expression was observed in a subset of primary colorectal cancers, usually in poorly differentiated colorectal cancers [30]. The significance of alteration of CDX2 expression in human cancers remains unclear, and therefore it is important to define the target genes which are downstream of CDX2. We have identified several CDX2-regulated genes such as *CDH17* (which encodes LI-cadherin) [24], *HEPH* (which encodes hephaestin) [31], *ABCB1* (which encodes multidrug resistance 1) [32], and *DSC2* (which encodes desmocollin 2) [33]. Among these genes, *ABCB1* was originally identified as an overexpressed and amplified gene in multiple drug-resistant cells, and its product, P-glycoprotein, seems to play a critical role in drug resistance [34]. Previously, we reported that forced expression of Reg IV in GC cells inhibited 5-FU-induced apoptosis through induction of Bcl-2 and dihydropyrimidine dehydrogenase [8]. Taken together, it is possible that in intestinal phenotype GC, expression (or ectopic expression) of CDX2 induces Reg IV and multidrug resistance 1 expression, resulting in an increase in drug-resistance. In fact, it has been reported that postoperative chemotherapy is not beneficial for patients with intestinal phenotype GC [35].

Although our data support the view that CDX2 plays a role in regulating *REG4* transcription via binding to the promoter region, several findings indicate that CDX2 alone is not sufficient for activating *REG4* expression. In the present study, we generated a polyclonal population of MKN-7, TMK-1, HSC-44PE, and KATO-III cells which express high levels of CDX2 by infection with retroviruses carrying a full-length human CDX2 cDNA. However, overexpression of CDX2 failed to activate Reg IV expression. In GC cell lines, none of the cell lines with undetectable CDX2 protein expression had detectable *REG4* transcripts and protein. Therefore, CDX2 is required for Reg IV expression, but CDX2 alone is not sufficient for activating Reg IV expression. In the present study, nine GC cell lines were studied. The origins of the cell lines were as follows. The MKN-7, MKN-28, and MKN-74 cell lines were established from intestinal type GC. The TMK-1 and MKN-45 cell lines were established from diffuse type GC. The KATO-III, HSC-39, and HSC-44PE cell lines were established from signet ring cell carcinoma. The MKN-1 cell line was established from adenosquamous cell carcinoma. Because in intestinal metaplasia of the stomach, CDX2 and Reg IV expression are well correlated, GC cell lines established from diffuse type GC or signet ring cell carcinoma may not be suitable for the analysis of Reg IV induction by CDX2. In fact, Reg IV expression can be induced by CDX2 in cell lines derived from colon cancer in the present study. Furthermore, we showed that H3K27me3 levels on the *REG4* promoter region were high in MKN-1 and MKN-28 GC cell lines. These 2 cell lines lacked detectable expression of *REG4* although CDX2 protein expression was found. Therefore, H3K27me3 levels on the *REG4* promoter region may be high in MKN-7, TMK-1, HSC-44PE, and KATO-III cells, in which overexpression of CDX2 failed to activate Reg IV expression.

It has been reported that *REG4* mRNA expression was enhanced by stimulation with TGF-α, EGF, HGF, or bFGF through activation of the mitogen-activated protein kinase (MAPK) pathway [22]. Thus, it could be hypothesized that Reg IV is also regulated by downstream transcriptional factors of MAPK pathways. We performed *in silico* analyses of the *REG4* gene 5′-flanking region, and found at least one presumptive AP-1 consensus sequences (at −883 base pairs of *REG4* gene 5′-flanking region), which is a downstream transcriptional factor of MAPK signalling. In the present study, HSC-39 cells showed similar

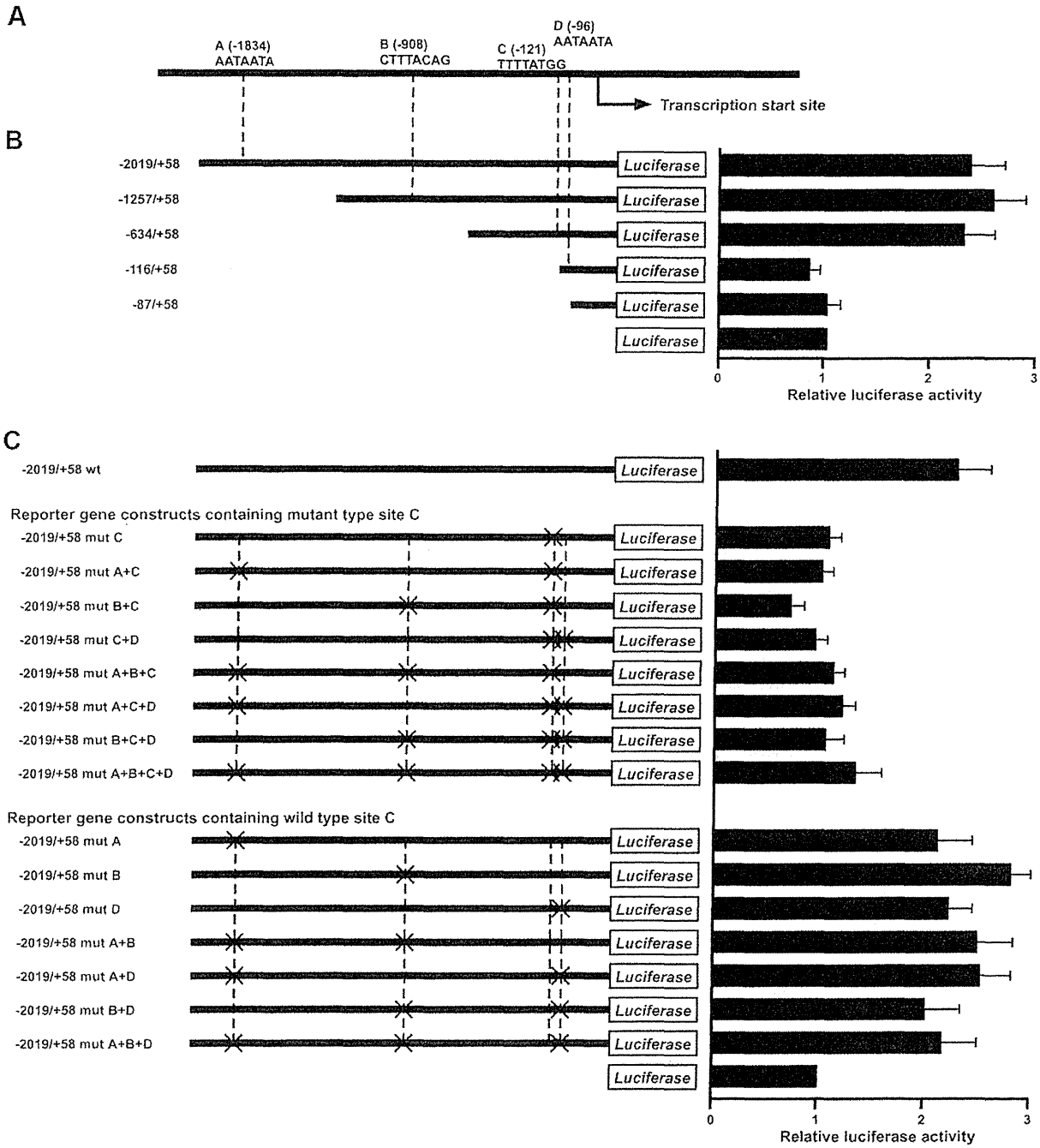

Figure 2. *REG4* promoter analysis. Localization of regulatory elements and CDX2 binding sites in the 5′-flanking region of the *REG4* gene. A: Schematic representation of the 5′-flanking region of the *REG4* gene. The location and sequence of 4 consensus CDX2-binding sites in the 5′-flanking region of *REG4* (i.e., sites A, B, C, and D) is indicated. B: Schematic representation of *REG4* reporter gene constructs. The *REG4* genomic DNA sequences present in the reporter gene vectors are indicated. Key sequences for *REG4* transcription reside between base pairs −634 and −116. Reporter assays with the series of *REG4* deletion constructs were performed in the CDX2-expressing GC cell line, HSC-39. The luciferase activity of the empty pGL4.10 basic vector was assigned a value of 1. The reporter assays were performed in triplicate, and mean and SD values of luciferase activity are shown. C: Localized mutations in the candidate CDX2-binding sites (i.e., sites A, B, C, and D) were introduced into the −2019/+58 construct, and the series of constructs generated is shown. The CDX2 candidate binding site designated as "C" plays critical roles in *REG4* transcription. Reporter assays were performed in CDX2-expressing GC cell line, HSC-39. The activity of the pGL4.10 basic vector was assigned a value of 1. Assays were performed in triplicate. Mean and SD luciferase activity values are shown.
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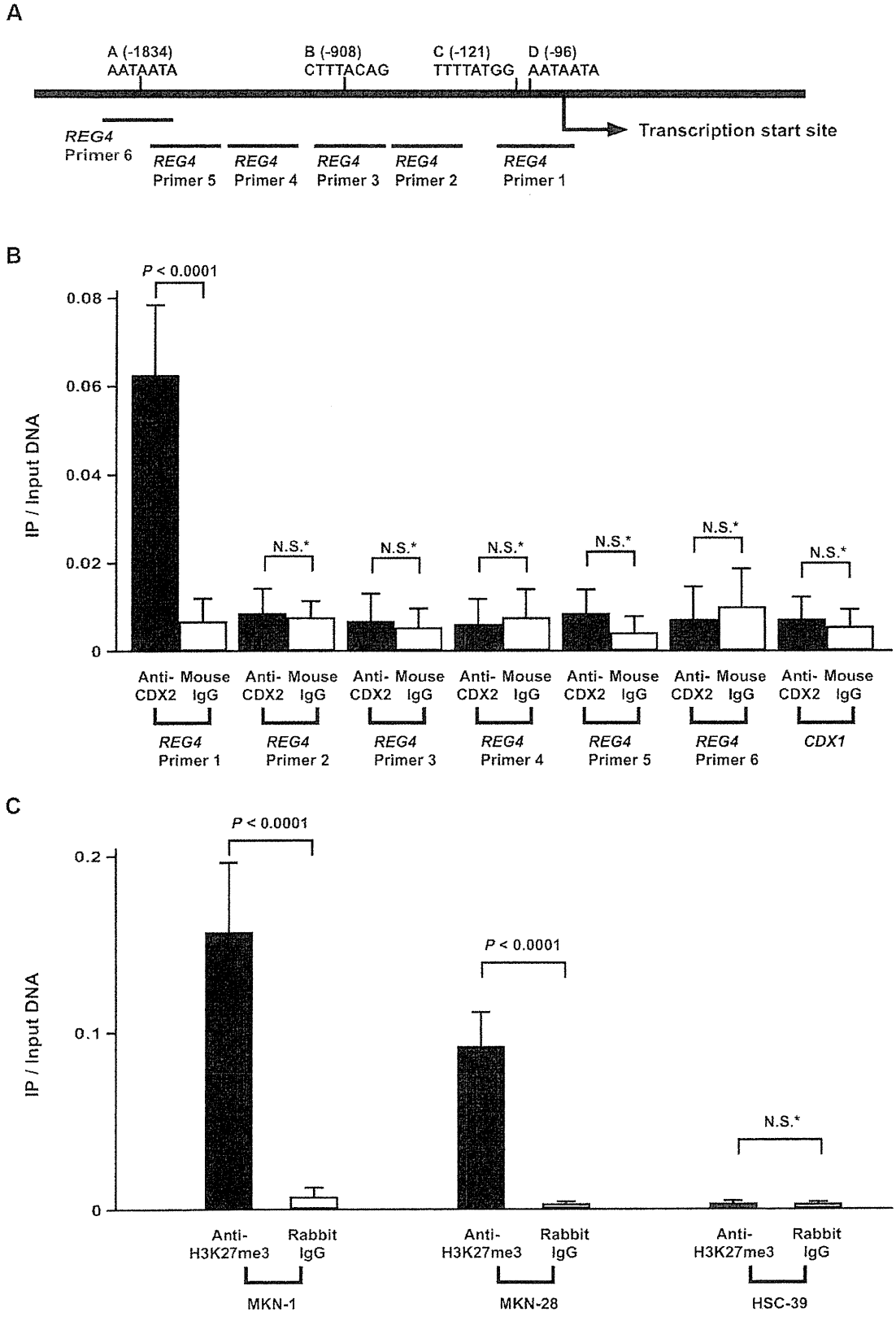
A
A (-1834)
AATAATA
B (-908)
CTTTACAG
C (-121)
TTTTATGG
D (-96)
AATAATA
Transcription start site
REG4
Primer 6
REG4
Primer 5
REG4
Primer 4
REG4
Primer 3
REG4
Primer 2
REG4
Primer 1
B
P < 0.0001
IP / Input DNA
0.08
0.06
0.04
0.02
0
N.S.*
Anti- CDX2
Mouse IgG
REG4 Primer 1
REG4 Primer 2
REG4 Primer 3
REG4 Primer 4
REG4 Primer 5
REG4 Primer 6
CDX1
C
P < 0.0001
IP / Input DNA
0.2
0.1
0
Anti- H3K27me3
Rabbit IgG
MKN-1
MKN-28
HSC-39

Figure 3. ChIP analysis of the 5′-flanking region of the *REG4* gene. A: Schematic representation of the 5′-flanking region of the *REG4* gene. The location of 4 consensus CDX2-binding sites in the 5′-flanking region of *REG4* (sites A, B, C, and D) and PCR primers (*REG4* Primer 1, 2, 3, 4, 5, and 6) are indicated. B: CDX2 binding to *REG4* promoter region shown by ChIP. Bulk (input) DNA was prepared as well as DNA isolated from ChIP with anti-CDX2 monoclonal antibody or mouse IgG. qPCRs were performed in triplicate for each sample primer set, and the mean and SD of the three experiments was calculated. C: ChIP analysis of H3K27me3 enrichment in the *REG4* gene promoter. ChIP enrichment was measured using qPCR. qPCRs were performed in triplicate for each sample primer set, and the mean and SD of the three experiments was calculated. *P* values were calculated using Student's t-test. * N.S. = not significant.
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transcriptional activity of reporter gene constructs containing 1.2 kb and 0.6 kb of *REG4* 5′-flanking sequence. As the effect of EGF or TGF-α on *REG4* transcription was not investigated in the present study, further investigation is needed to clarify the signalling mechanisms which induce regulation of *REG4* transcription.

In conclusion, our present data show that CDX2 protein directly regulates Reg IV expression. Reg IV activates the EGFR/Akt/AP-1 signaling pathway. As intestinal phenotype GC frequently expresses EGFR [36], it is suggested that this Reg IV-activated pathway plays an important role in this subtype of GC. Because CDX2 also induces expression of the multidrug resistance gene, *ABCB1*, anti-EGFR therapy but not chemotherapy may be beneficial for patients with intestinal phenotype GC.

Materials and Methods

Plasmids

The CDX2 cDNA was inserted into the multiple cloning site of the retroviral expression vector pPGS-CMV-CITE-neo as described previously [24]. The full-length, wild-type CDX2 cDNA was also subcloned into the retroviral vector pBabe-Puro ER as described previously to generate pCDX2-ER [24]. The pCDX2-ER vector encodes a chimeric protein in which full-length CDX2 sequences are fused upstream of a mutated ER ligand–binding domain. The mutated ER ligand-binding domain no longer binds estrogen, but retains the ability to bind tamoxifen. Genomic DNA sequences from the 5′-flanking region of the human *REG4* gene were amplified by PCR using genomic DNA purified from HSC-39 cells as a template and subcloned into the pGL4.10 [luc2] vector (Promega, Madison, WI). PCR-based approaches were used to introduce mutations into the presumptive CDX2-binding sites in the pGL4.10-REG4 reporter gene construct using QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Four putative CDX2-binding sites were changed. All fragments generated by PCR were verified by automated sequencing. The plasmid pGL4.74 [hRluc/TK] vector (Promega) was used as a control for transfection efficiency in reporter assays.

Cell Lines, Retrovirus Infections, and Drug Treatment

The amphotropic Phoenix packaging cell line was provided by G. Nolan (Stanford University, Stanford, CA) [37]. Nine cell lines derived from human GC and 2 cell lines derived from human colon cancer were used. The TMK-1 cell line was established in our laboratory [38]. The HSC-39 and HSC-44PE cell lines were established by one of the authors (Kazuyoshi Yanagihara) [39,40]. Five GC cell lines of the MKN series were kindly provided by Dr. Toshimitsu Suzuki [41,42]. The KATO-III cell line was kindly provided by Dr. Morimasa Sekiguchi [43]. The HT-29 and SW480 colon cancer cell lines were obtained from the American Type Culture Collection. Cells were stored in liquid nitrogen until the initiation of this study. After thawing from frozen stock, the cells were kept at low passage throughout the study. Consistent cell morphology was monitored by comparison of microscopic images. The Phoenix packaging cells were transfected with retroviral expression constructs (pPGS-CDX2, pPGS-neo, and pCDX2-ER) and the supernatant containing nonreplicating amphotropic virus was harvested as previously described [24]. In HT-29 cells expressing the CDX2-ER fusion protein (HT-29/CDX2-ER), CDX2 function was activated by addition of 4-hydroxytamoxifen (4-OHT) (Sigma Chemical, St. Louis, MO) to the growth medium at a final concentration of 500 nmol. To investigate whether DNA methylation induced transcriptional inactivation of Reg IV, cells were treated with a final concentration of 1 μM Aza-dC (Sigma Chemical) for 5 days before they were harvested for RNA extraction.

Western Blot Analysis

For Western blot analysis, cells were lysed as described previously [44]. Protein concentrations were determined by Bradford protein assay (BioRad, Richmond, CA) with BSA used as the standard. The lysates (20 μg) were solubilized in Laemmli's sample buffer by boiling and then subjected to 12% SDS-polyacrylamide gel electrophoresis followed by electro-transfer onto a nitrocellulose filter. The filter was incubated for 1 hour at room temperature with an anti-Reg IV antibody (rabbit polyclonal antibody developed in our laboratory, Ref. 10) or anti-CDX2 antibody (BioGenex, San Ramon, CA). Peroxidase-conjugated anti-rabbit or anti-mouse IgG was used in the secondary reaction. Immunocomplexes were visualized with an ECL Plus Western Blot Detection System (Amersham Biosciences, Piscataway, NJ). β-actin (Sigma Chemical) was also detected as a loading control.

qRT-PCR Analysis

Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA), and 1 μg of total RNA was converted to cDNA with a First Strand cDNA Synthesis Kit (Amersham Biosciences). Quantitation of *REG4* mRNA levels was performed by real-time fluorescence detection as described previously [45]. PCR was performed with a SYBR Green PCR Core Reagents Kit (Applied Biosystems, Foster City, CA). Real-time detection of the emission intensity of SYBR green bound to double-stranded DNA was performed with an ABI PRISM 7900 Sequence Detection System (Applied Biosystems) as described previously [46]. *ACTB*-specific PCR products were amplified from the same RNA samples and served as an internal control. Sequences of primers for *REG4* qRT-PCR are shown in **Table 1**. qRT-PCRs were performed in triplicate for each sample primer set, and the mean and standard deviation (SD) of the three experiments was calculated as the relative quantification value. At the end of 40 PCR cycles, reaction products were separated electrophoretically on 8% non-denaturing polyacrylamide gels for visual confirmation of PCR products.

RNAi

To knockdown the endogenous CDX2, RNAi was performed. Two siRNA duplexes targeting CDX2 (5′-AACCAGGACGAAAGACAAAUA-3′, CDX2 siRNA1; and 5′-AAGCCUCAGUGUCUGGCUCUG-3′, CDX2 siRNA2) and a nonsilencing siRNA duplex (5′-AAUUCUCCGAACGUGUCACGU-3′) were synthesized (Qiagen). Transfection was performed using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the manu-

Table 1. Primer sequences for qRT-PCR and ChIP assay.

Sense	Anti-sense
qRT-PCR for *REG4*	
5′-GCCCCGCCATCCCTT-3′	5′-CTGCTCGAGACAGCCAGAGA-3′
qRT-PCR for *ACTB*	
5′-TCACCGAGCGCGGCT-3′	5′-TAATGTCACGCACGATTTCCC-3′
ChIP for *REG4* (Primer 1)	
5′-GGAGAGGTTCTTTTCCTGGCTAG-3′	5′-GCAACCAAGACTCTAAGGGCC-3′
ChIP for *REG4* (Primer 2)	
5′-CCCTTTGCCATCTATACTGGAAA-3′	5′-CATTACACACTCAAGAAACCCAACC-3′
ChIP for *REG4* (Primer 3)	
5′-TCAGCTTCACCCACAACTGTCT-3′	5′-TTAGGTTGTAGGTGCCAGAGATGA-3′
ChIP for *REG4* (Primer 4)	
5′-CAAAGGTTTATGTGAGTCCTATCAATG-3′	5′-CCTGTGTTTCCAGCAGCCAT-3′
ChIP for *REG4* (Primer 5)	
5′-CTATTCGAAAGCTGCCTGGC-3′	5′-AAATTGTCTGAATCAAAAAGGTCCA-3′
ChIP for *REG4* (Primer 6)	
5′-GCAGGAGATAAAAGGCTACACGTT-3′	5′-GGAGAGATAAAGTGGAAGCCAGG-3′
ChIP for *CDX1*	
5′-TCCTCGTCTCTCCTTCTTGC-3′	5′-AGAAGGTCAGGGCTGAGACTC-3′

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facturer's protocol. Briefly, 60 pmol of siRNA and 10 μL of Lipofectamine RNAiMAX were mixed in 1 mL of RPMI medium (10 nmol/L final siRNA concentration). After 20 min of incubation, the mixture was added to the cells and these were plated on dishes for each assay. Three days after transfection, cells were analyzed for all experiments.

Reporter Gene Assays

HSC-39 and MKN-1 cells were seeded in 6-well plates (BD Falcon, Franklin Lakes, NJ). Transfection of cells at 50%–80% confluency was performed with 3 μL of FuGENE6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN), 0.8 μg of pGL4.10 reporter gene constructs, and 0.2 μg pGL4.74 [hRluc/TK] vector (Promega). At 48 hours after transfection, cells were collected and resuspended in passive lysis buffer (Promega). Luciferase activity was determined with a dual luciferase assay system (GloMax 96 Microplate Luminometer, Promega).

ChIP Assays

The ChIP assays were performed using the EZ-ChIP Chromatin Immunoprecipitation Kit (Millipore, Billerica, MA) per manufacture instructions. To analyze whether CDX2 directly binds to the putative CDX2-binding sites in the *REG4* 5′-flanking region, we performed ChIP assays using HSC-39 cells. In brief, HSC-39 cells ($1–2\times10^7$) were cross linked with 1% formaldehyde in phosphate buffered saline (PBS) for 15 min at 37°C, and glycine was added to quench reactive aldehydes. After washing cells with cold PBS, cells were resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris pH 8.1) with Proteinase Inhibitor (Roche Diagnostics). After samples were sonicated, chromatin extracts containing DNA fragments (average size, 500 base pairs) were immunoprecipitated using 2 μg monoclonal anti-CDX2 antibody (BioGenex) or 2 μg mouse IgG (Millipore). Each immunoprecipitated DNA sample was quantified by qPCR using primers listed in **Table 1**. As a negative control, an approximately 200 base pairs DNA fragment from exon 3 of the *CDX1* gene was amplified by PCR using specific primers (**Table 1**).

To determine the enrichment of H3K27me3 on the *REG4* promoter in the GC cell lines, ChIP assays were performed using MKN-1, MKN-28, and HSC-39 cell lines. In brief, GC cells ($1–2\times10^7$) were cross linked with 1% formaldehyde in PBS for 15 min at 37°C, and glycine was added to quench reactive aldehydes. After washing cells with cold PBS, cells were resuspended in SDS lysis buffer with Proteinase Inhibitor (Roche Diagnostics). After samples were sonicated, chromatin extracts containing DNA fragments (average size, 500 base pairs) were immunoprecipitated using 2 μg polyclonal anti-H3K27me3 antibody (Abcam, Cambridge, MA) or 2 μg rabbit IgG (Millipore). Each immunoprecipitated DNA sample was quantified by qPCR using *REG4* Primer 1 (**Table 1**).

qPCRs were performed in triplicate for each sample primer set, and the mean and standard deviation (SD) of the three experiments was calculated as the relative quantification value. At the end of 40 PCR cycles, reaction products were separated electrophoretically on 8% non-denaturing polyacrylamide gels for visual confirmation of PCR products.

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Author Contributions

Conceived and designed the experiments: YN NO TH NS KS HO KY HS WY. Performed the experiments: YN NO NS. Analyzed the data: YN NO NS. Contributed reagents/materials/analysis tools: TH HO KY HS. Wrote the paper: YN NO WY.

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The Search for Secreted Proteins in Prostate Cancer by the *Escherichia coli* Ampicillin Secretion Trap: Expression of NBL1 Is Highly Restricted to the Prostate and Is Related to Cancer Progression

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CAST · NBL1 · Prostate cancer

Abstract
Aims: Genes expressed only in cancer tissue or specific organs will be useful molecular markers. To identify genes that encode secreted proteins present in prostate cancer (PCa), we generated *Escherichia coli* ampicillin secretion trap (CAST) libraries from PCa and normal prostate (NP). ***Methods and Results:*** We identified 15 candidate genes that encode secreted proteins present in PCa and NP. Quantitative RT-PCR analysis revealed that *MSMB*, *NBL1* and *AZGP1* were expressed with much higher specificity in PCa and NP than in 14 other kinds of normal tissue. We focused on *NBL1*, which was originally identified as a putative tumor suppressor gene. Western blot analysis revealed that NBL1 protein was highly expressed in both cell lysate and culture media of the DU145 PCa cell line. Immunohistochemical analysis showed that NBL1 expression was highly detected in and restricted to NP and PCa and was significantly down-regulated in PCa. NBL1 expression was significantly reduced according to the tumor stage, Gleason grade and preoperative prostate-specific antigen (PSA) value. ***Conclusion:*** NBL1 is a secreted protein that is highly restricted to the prostate. Underexpression of NBL1 correlated with PCa progression. NBL1 might be a candidate tumor marker for PCa in addition to PSA.

Introduction

Prostate cancer (PCa) is one of the most common human male cancers. Cancer develops as a result of multiple genetic and epigenetic alterations [1]. Better knowledge of changes in gene expression that occur during prostatic carcinogenesis may lead to improvements in its diagnosis, treatment and prevention [2]. Genes encoding secretory proteins expressed specifically in cancers or specific organs may be ideal biomarkers for cancer diagnosis. To identify novel genes that encode secreted protein present in PCa, we used the *Escherichia coli* ampicillin secretion trap (CAST) method. We have previously identified several PCa-specific genes encoding transmembrane proteins with the CAST method and reported their high potential as therapeutic targets [3, 4]. To our knowledge, however, CAST analysis of secreted protein in PCa has not been reported.

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Prostate-specific antigen (PSA), a serine protease found in semen, is the most widely used serum marker for detecting and monitoring PCa [5, 6]. The rapid incorporation of aggressive PSA testing has resulted in dramatically earlier identification of PCa and is attributed with the decrease in mortality from PCa [7]. However, there are limitations to the use of PSA. PSA levels are also increased in benign prostatic hyperplasia (BPH) and general inflammatory responses. PSA testing has the potential disadvantage of low specificity and has led to a tremendous increase in the number of unnecessary prostate biopsies [8]. Furthermore, blood PSA level is not significantly increased in patients with poorly differentiated PCa and patients receiving androgen deprivation therapy [9, 10]. Moreover, the prognosis of castration-resistant PCa remains unsatisfactory [11]. Therefore, increasing emphasis has been placed on the need to determine new protein biomarkers for use in the diagnosis of PCa.

In the present study, to identify genes that encode secreted proteins, we generated CAST libraries from 2 PCa cell lines, LNCaP and DU145, and normal prostate (NP). CAST is a signal sequence trap method developed by Ferguson et al. [12]. Signal peptides target secreted and transmembrane proteins to their appropriate subcellular localization [13]. A consensus sequence for the signal peptide has not been identified, and, thus, standard molecular techniques are not well suited to identify such proteins. CAST exploits the ability of mammalian signal sequences to confer ampicillin resistance to a mutant β-lactamase lacking the endogenous signal sequence [14]. We report here the identification of several genes that encode secreted proteins expressed in PCa and NP. Among these, we focused on the *NBL1* gene because this gene is highly restricted in PCa and NP. *NBL1* (neuroblastoma suppression of tumorigenicity 1) was originally identified as a putative tumor suppressor gene in a transformed fibroblast rat model [15]. We confirmed the expression of NBL1 in cell lysate and culture media of PCa cells. We examined the expression and distribution of NBL1 in human PCa and NP by immunohistochemistry and compared them with clinicopathological characteristics. We also studied the expression of NBL1 in normal systemic organs because NBL1 has the possibility of being a serum marker for PCa.

Materials and Methods

CAST Library Construction

Plasmid CAST (pCAST) was designed to contain the kanamycin resistance gene and the β-lactamase gene lacking the first 69 nucleotides encoding the endogenous signal peptide. *Eco*RI and *Bam*HI sites were placed upstream of the mutant β-lactamase gene for directional cloning. CAST library construction was performed as described previously [3, 12]. In brief, CAST cDNA libraries were generated from 2 μg of mRNA with a random primer containing a *Bam*HI restriction site for reverse transcription (SuperScript Choice System; Invitrogen, Carlsbad, Calif., USA). The *Eco*RI-adapted cDNA was digested with *Bam*HI, size fractionated, ligated into pCAST and plated onto Luria-Bertani/ampicillin medium. Individual colonies were picked and grown in 1.0 ml of this medium with kanamycin in a 96-well format. Plasmid DNA was sequenced in a 96-well format using a primer located within the β-lactamase gene.

Table 1. Characteristics of the PCa patients (n = 181)

Age	<70 years	92 (51%)
	≥70 years	89 (49%)
PSA at diagnosis	<20	108 (60%)
	20 to <100	31 (17%)
	≥100	42 (23%)
Gleason score	6	64 (35%)
	7	44 (24%)
	8	40 (22%)
	9–10	33 (18%)
pStage	B	57 (31%)
	C	79 (44%)
	D	45 (25%)
Treatment	prostatectomy	127 (70%)
	hormone and/or radiation	54 (30%)

Tissue Samples

In total, 219 primary tumor samples were collected from patients diagnosed with PCa and those with NP. Patients were treated at the Hiroshima University Hospital or an affiliated hospital. Because written informed consent was not obtained, for strict privacy protection, identifying information for all samples was removed before analysis. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government.

For quantitative RT-PCR, 16 PCa samples and 9 non-neoplastic samples were used. Samples were frozen immediately in liquid nitrogen and stored at −80°C until use. We confirmed microscopically that the tumor specimens consisted mainly (>50%) of cancer cells. Noncancerous samples of heart, lung, stomach, small intestine, colon, liver, pancreas, kidney, bone marrow, peripheral leukocytes, spleen, skeletal muscle, brain and spinal cord were purchased from Clontech (Palo Alto, Calif., USA).

For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from 127 PCa patients treated by radical prostatectomy, 54 PCa patients who underwent prostate biopsy, and 13 patients treated by suprapubic prostatectomy for BPH. Tumor staging was in accordance with the TNM classification system [16], and histological classification of PCa was made in accordance with the World Health Organization classification [17]. The clinical characteristics of the PCa patients are shown in table 1. In addition, we used archival formalin-fixed, paraffin-embedded tissues from normal systemic organs including 5 speci-

Table 2. List of genes encoding secreted protein from the CAST libraries

	Sample name		
	LNCaP	DU145	Normal Prostate
Genes encoding secreted protein	6	8	7
Gene name	TFPI	TFPI	MSMB
	SPP1	CCDC126	AZGP1
	FN1	CLU	SPP1
	COL4A5	DMKN	TFPI
	CALU	NBL1	C1RL
	SFTPA1B	ARMETL	CLU
		FGB	SRGN
		NTN4	

mens each of the brain, spinal cord, heart, lung, esophagus, stomach, small intestine, colon, liver, spleen, pancreas, kidney, adrenal, ureter, bladder, testis, skin, skeletal muscle and blood vessels.

Quantitative RT-PCR and Western Blot Analysis

Quantitative RT-PCR was performed with an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, Calif., USA) as described previously [18]. Quantitation of *NBL1* mRNA levels was done by real-time fluorescence detection as reported in a previous study [19]. The *NBL1* primer sequences were 5′-TCAACAAGCTGGCACTGTTC-3′ and 5′-GCAGGAGTCACAGTGAACCA-3′ (for more information, see online suppl. material at www.karger.com/doi/10.1159/000341396).

For Western blot analysis, tissue samples or cells were lysed as described previously [20]. The primary antibody against NBL1 (R&D Systems, Inc., Minneapolis, Minn., USA) was used (see online suppl. material).

Evaluation of Specificity of Gene Expression

To evaluate the specificity of expression in each gene, a specificity index was calculated as follows: first, we identified the 14 normal tissues, which are indispensable for survival, in which the target gene expression was highest among tissues analyzed by quantitative RT-PCR. We then identified PCa among the 16 PCa samples in which the target gene expression was highest by quantitative RT-PCR (mRNA expression level in this tissue was denoted as A). We next identified NP among the 9 NP samples in which the target gene expression was highest by quantitative RT-PCR (mRNA expression level in this tissue was denoted as B). The target mRNA expression levels were standardized to normal organ with highest expression set as 1. A and B were defined as the PCa and NP specificity indices, respectively. When the specificity index of the target gene in A and/or B was ≥ 10, the gene was considered to show high specificity. When the specificity index of the target gene was <10 and ≥ 5, the gene was considered to show low specificity. When the specificity index of the target gene was <5, the gene was considered to show no specificity.

Immunohistochemistry

Immunohistochemical analysis was performed with a Dako Envision+ mouse peroxidase detection system (Dako Cytomation, Carpinteria, Calif., USA). The following antibody dilutions were used: goat polyclonal anti-NBL1, 1:100 (R&D Systems; see supplementary material). We used the percentage of NBL1-positive cells in the total cells as an immunohistochemical scoring system. Evaluation of immunoreactivity was scored independently by two pathologists (K.S. and T.H.). The sections from the 127 patients treated by radical prostatectomy were evaluated in both cancerous and non-cancerous areas. The sections from the 54 prostate biopsy patients were evaluated in the cancerous area. The percentage of NBL1 expression in each region was scored as follows: no expression, 0; <10%, 1+; 10–30%, 2+; 31–50%, 3+, and >50%, 4+. We used NBL1 expression in nerve cells as a positive control based on previously reported NBL1 immunoreactivity in nerve cells [21].

RNA Interference and Cell Growth and in vitro Invasion Assays

RNA interference was performed to knock down the endogenous *NBL1*. siRNA oligonucleotides for *NBL1* and a negative control were purchased from Invitrogen (Carlsbad, Calif., USA). The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and modified Boyden chamber assays were performed to examine cell growth and invasiveness, respectively (see online suppl. material) [22, 23].

Statistical Method

Associations between clinicopathological variables and NBL1 expression were analyzed by Mann-Whitney U test. The comparison of cell growth and invasive activity was analyzed by t test. A value of $p < 0.05$ was considered statistically significant.

Results

Identification of Genes with Higher Expression in the Prostate than in 14 Normal Tissues

To identify genes that encode secreted proteins present in PCa, we generated CAST libraries from 2 PCa cell lines (LNCaP and DU145) and NP, as previously described [3, 4]. We identified 6, 7 and 8 genes encoding secreted proteins from the respective cell lines and NP. The names of these 21 genes are shown in table 2. We performed quantitative RT-PCR to identify genes expressed specifically in PCa and NP. Representative results are shown in figure 1. We then identified the PCa among 16 tissues in which the target gene expression was highest (mRNA expression levels are shown as A; table 3) and NP among 9 tissues in which the target gene expression was highest (mRNA expression levels are shown as B; table 3). Next, the PCa specificity index (A/B ratio) for each gene was calculated. We could not find a gene specific only to PCa, but we could detect genes specific to both PCa and NP. Because