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Twist1 causes the transcriptional repression of claudin-4 with prognostic significance in esophageal cancer

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ABSTRACT

Twist1 is a transcription factor that is involved in epithelial–mesenchymal transition by suppressing intercellular adhesion. In this study, we aimed to determine the role of Twist1 in the regulation of claudin-4 expression and investigate its specific mechanisms and clinical implications using human esophageal carcinoma cell lines and tissues. As a result, up-regulation of Twist1 decreased both gene and protein expression levels of endogenous claudin-4 and the suppression was mediated by direct binding of Twist1 to the canonical E-box in the promoter region of claudin-4. In addition, there was a significant inverse correlation of claudin-4 with Twist1 in esophageal cancer tissues. High Twist1 and low claudin-4 expression was associated with the poorest prognosis and was more highly correlated with adverse outcome than any other subgroup with statistical significance ($p = 0.001$). Our results indicate that Twist1 induces the repression of claudin-4 expression during the epithelial–mesenchymal transition in esophageal carcinoma.

1. Introduction

It is widely recognized that the loss of cell-to-cell adhesion due to dysfunction or diminution of the tight junctions (TJs)-related molecules is one of the crucial events in the process of tumor progression and invasion, allowing the release of individual carcinoma cells from the primary tumor. As with junctional molecules such as E-cadherin, decreased levels of claudins have been detected in human tumors, and this type of reduction eventually increases the grade of malignancy [1]. It has been increasingly reported that loss of cell polarity with disruption of TJs is related to the epithelial–mesenchymal transition (EMT) that is involved in cancer metastasis [2,3]. A core signature in EMT was derived from the changes mediated by up-regulation of transcription factors such as Goosecoid, Snail, Slug, and Twist, and by down-regulation of TJ-related proteins, the loss of which can trigger EMT [4].

The Twist protein is a highly conserved transcription factor belonging to the basic helix-loop-helix protein family [5]. So far, two Twist genes, Twist1 and Twist2, have been reported to be involved in the regulation of developmental and morphogenetic events for cellular differentiation in vertebrates [6]. To date, several reports indicate that Twist1 is a key transcription factor that induces cancer metastasis via the regulation of gene expression of proteins closely associated with the initiation of EMT [7,8]. For example, Twist1 can directly or indirectly suppress the expression of E-cadherin, a representative protein of the adherence junction, and cause cancer progression and metastasis in most types of human malignancies [7,9]. In addition, Twist1 was observed to be inversely related with levels of other TJ-related proteins, such as claudins, in some cancer tissues [10,11]. However, little is known about the detailed mechanism underlying the regulation of these TJ-related proteins by Twist1.

The claudins are a recently identified integral transmembrane protein family that is mainly responsible for the intercellular adhesion and permeability of TJs [12]. The expression of claudins has been found to be abnormally altered in several cancers in a tissue-specific manner. Among the various claudin subtypes, increased claudin-4 (CLDN4) expression is associated with malignant behavior in ovarian, breast, and pancreatic cancers [13–15]. On the other hand, a loss of CLDN4 expression appears to be closely associated with aggressive behavior and development in a variety of cancers including colorectal, gastric, and esophageal cancers [16–18]. Interestingly, decreased expression of CLDN4 in some gastrointestinal

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cancers results in higher malignancy grades in terms of potential invasiveness and metastatic ability [16,19,20]. As of yet, there have been few studies on the repression of CLDN4 by transcriptional regulation and no previous studies on the correlation between Twist1 and CLDN4 expression in esophageal carcinoma.

Previously, we reported that down-regulation of CLDN4 is associated with poor prognosis in esophageal carcinoma [18] and that over-expressed Twist1 is also a significant adverse prognostic factor and strongly related with EMT in the same cancer [21]. Based on these previous clues, we hypothesized that Twist1 might influence the regulation of CLDN4 expression as a possible transcriptional repressor. Then we investigated a significant inverse relationship between Twist1 and CLDN4 in esophageal cancer cell lines and tissues and analyzed their clinical implications.

2. Materials and methods

2.1. Cells and plasmids

Human esophageal carcinoma cell line TE8 and TE10 were purchased from RIKEN (Saitama, Japan). The pSG5.hTwist1.HA vector was generated by insertion of full-length human Twist1 gene amplified by a Twist1 primer pair (Supplementary Table 1). pGL3 and the pGL3.C4P1 vectors were kindly provided by Dr. Pat J. Morin from the NIH, and pGL3.C4P2 vector was constructed by inserting a truncated CLDN4 promoter region (−322 to +42 bp).

2.2. Quantitative real-time RT-PCR

Total RNA was isolated using an Easy-Spin RNA Extraction Kit (iNtRON, Korea) from cell lysates. Then, cDNA was synthesized from 0.1 μg of total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions. The mRNA expression levels of the target genes and GAPDH as a normalizing control were analyzed with an ABI 7900 HT Fast Real-Time PCR system (Applied Biosystems) using specific primers (Supplementary Table 1). Each measurement was performed in duplicate and PCR product quality was monitored using post-PCR melting curve analysis. All of the relative mRNA expressions were determined by calculating the $2^{-\Delta\Delta CT}$ based on analyzed C_T values [22].

2.3. Western blot analysis

Cell lysates were prepared and electro-transferred from the gels to nitrocellulose membranes after SDS–PAGE. Subsequently, the membranes were blocked with 5% skim milk solution for 1 h and incubated with antibodies against Twist1 (Abcam, UK), claudin-4 (Acris, Germany), and α-tubulin (Santa Cruz Biotechnology Inc., USA) followed by HRP-linked secondary IgG (sc2004 or sc2005; Santa Cruz Biotechnology Inc.). The target proteins were visualized using the Enhanced Chemiluminescence System (Amersham Biosciences, Germany).

2.4. Lentivirus transduction and transient transfection

pHR.CMV.FLAG-hTwst1-IRES-Hygro vector was generated by the insertion of human Twist1 gene into pHR.CMV.FLAG-IRES-Hygro. For the virus package, pHR.CMV.FLAG-hTwist1-IRES-Hygro and pHR.CMV.GFP-IRES-Hygro vectors were transfected into 293T cells by Lipofectamine 2000 (Invitrogen). Cells were transduced with each virus concentrate supplemented with polybrene (Millipore, USA), and transfectants were selected for using Hygromycin. The pGL3.C4P1 vector was transfected into cultured cells and the pSG5.hTwist1.HA vector and/or control vector were transiently co-transfected into each of the cell types.

2.5. Luciferase reporter assay

TE8 and TE10 cells were plated into 24-well plates and incubated for 24 h. Promoter-reporter vectors were co-transfected with either pSG5.hTwist1.HA or the pSG5.HA control vector into cells. Luciferase activities were measured using the Luciferase Assay System E1501 (Promega, USA) according to the manufacturer's instructions, and the bacterial ß-galactosidase gene (pCMV-ßgal) was used as a control for transfection efficiency.

2.6. Chromatin immunoprecipitation (ChIP) analysis

TE8 cells were grown to 70–80% confluence, fixed with 1% formaldehyde, and then lysed for nuclear extraction. Nuclear extracts were then sonicated to shear the DNA to lengths less than about 500 bp. Twist1-DNA complexes were immunoprecipitated using Protein G Dynabeads (Invitrogen) conjugated with anti-Twist1 IgG sc81417 or control mouse IgG (Santa Cruz Biotechnology Inc.). After the precipitated DNA was eluted, crosslinks were reversed with 0.3 M NaCl and the DNA was purified using QIAquick PCR Purification Kit (Qiagen, USA). Then, quantitative PCR was performed on the purified DNA using the primer sets listed in Supplementary Table 1.

2.7. Site-directed mutagenesis

The Twist1-binding E-box site (CAGCTG) on the CLDN4 promoter was mutated using pGL3.C4P2 as the template. The PCR for site-directed mutagenesis was performed with C4P2.E3M and C4P2.E5M primer sets to construct three- and five-base pair mutants in an E-box region, respectively.

2.8. Patients and tissue samples

We investigated 198 cases of esophageal squamous cell carcinomas (ESCC). The data were procured from surgical pathology files maintained in the pathology department of Seoul Samsung Medical Center, Korea. All archival materials were routinely fixed in 10% neutral-buffered formalin and embedded in paraffin.

2.9. Immunohistochemistry

Immunostaining for the Twist1 and CLDN4 protein was performed using an anti-Twist1 mouse monoclonal antibody (Twist2-C1a; Abcam) and an anti-claudin-4 rabbit monoclonal antibody (3E2C1; Zymed Laboratories Inc., CA, USA), respectively. Tissue sections embedded in the microslides were deparaffinized with xylene, hydrated in alcohol, and immersed in Dako REAL™ peroxidase-blocking solution (DAKO Inc., Denmark). The sections were microwaved in 10 mM Tris buffer (pH 9.0) for 15 min, then mouse- or rabbit-specific secondary antibodies (Dako REAL™ EnVision™/HRP) and detection reagents were applied.

2.10. Evaluation of immunohistochemical staining

We used a scoring method to evaluate both the intensity of immunohistochemical staining and the proportion of stained epithelial cells. Staining intensity was classified as (i) weak, (ii) moderate, or (iii) strong. Positive cells were quantified as a percentage of the total number of epithelial cells and were assigned a point value corresponding to one of five categories: 0, <5%; 1, 5–25%; 2, 26–50%; 3, 51–75%; and 4, >75%. The percent positivity in the tumor cells and the staining intensities were then multiplied to

generate the immunohistochemistry score for each tumor specimen. For the categorical analyses, the immunoreactivity in tumor cells was graded as low (total score <4) or high (total score ⩾4).

2.11. Statistical analysis

Overall survival was determined using the Kaplan–Meier method, and survival curves were compared using the log-rank test. Survival was measured from the date of surgery. All tests were two-sided and *P*-values less than 0.05 were regarded as statistically significant. Statistical analysis was performed using SPSS (version 17, IL, USA).

3. Results

3.1. Twist1 down-regulates CLDN4 expression in an esophageal cancer cell line

We investigated whether Twist1 is involved in repression of CLDN4, possibly as a transcriptional repressor. First, we transduced TE8 cells with either Twist1 or control GFP lentivirus and analyzed the mRNA and protein expression levels of Twist1 and endogenous CLDN4. As a result, we found that as mRNA expression of Twist1 was increased by infection with Twist1 lentivirus (Fig. 1A), the mRNA level of CLDN4 was decreased, and this suppression was detected in the protein levels as well (Fig. 1B). Transient transfection of the pSG5.hTwist1.HA vector also led to down-regulation of CLDN4 expression in a dose-dependent manner in the cell line (Fig. 1C).

To assess the transcriptional role of Twist1 in the regulation of CLDN4, we further performed luciferase reporter assays using the pGL3.C4P1 vector carrying the CLDN4 promoter region. For this study, both the pGL3.C4P1 and pSG5.hTwist1.HA vectors were transiently co-transfected into TE8 cells. As shown in Fig. 1D, the CLDN4 promoter-reporter activity was significantly reduced when Twist1 was introduced into the cells. These results show that up-regulated Twist1 could specifically down-regulate CLDN4 at the transcriptional level via an interaction with the promoter region of CLDN4 in esophageal cancer cells.

3.2. Twist1 binds to an E-box site of the CLDN4 promoter

In order to determine the mechanism underlying the Twist1-induced down-regulation of CLDN4 expression, we investigated whether Twist1 directly binds to the CLDN4 promoter. First, CLND4 promoter sequences of several mammals, including humans, were collected and aligned to find the conserved E-box sequence to which Twist1 would be expected to bind. We found an E-box site conserved throughout the analyzed mammals within the proximal region of the CLDN4 promoter (Fig. 2A). To assess the specific binding of Twist1 to this locus, we performed ChIP assays using TE8 cells that had been induced to express a high level of Twist1. Specific primer sets for a known Twist1-binding E-box CACATG (PDGFRα; −1839 to −1834 bp) in the PDGFRα promoter as a positive control [23], the conserved proximal E-box CAGCTG (C4PTE; −261 to −256 bp), and a negative control site (C4PTC; −10899 to −10807 bp) in the CLDN4 promoter were designed as shown in Fig. 2B. After ChIP with anti-Twist1 and control IgG antibody, the binding of Twist1 to each locus was quantitatively determined by real-time PCR, and Twist1 showed a significant binding to the conserved proximal E-box CAGCTG when compared with the IgG control (Fig. 2C). Twist1 strongly bound to the known Twist1-binding E-box in the PDGFRα promoter and no amplification was detected in the control site of the distal region in the CLDN4 promoter that does not include an E-box sequence. These results indicate that Twist1 binds directly or as part of a complex to the endogenous CLDN4 promoter.

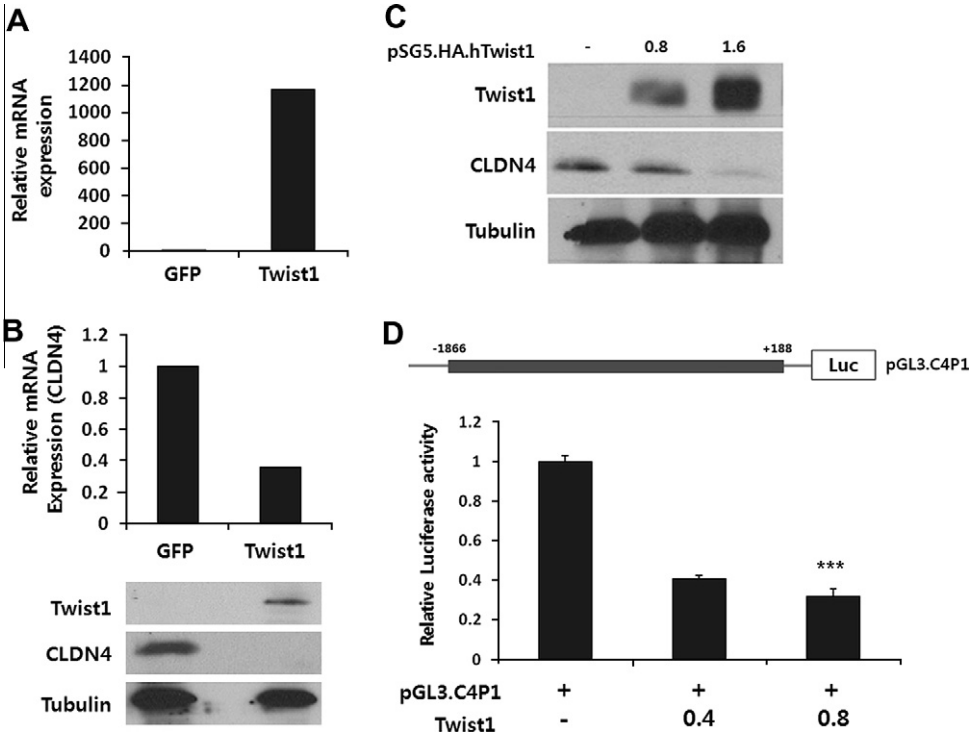

Fig. 1. Twist1 expression is inversely correlated with CLDN4 expression in esophageal cancer cells. (A) Lentiviral transduction of Twist1 was confirmed by real-time RT-PCR of Twist1-introduced TE8 cells. (B) Twist1 up-regulated by transduction inhibited mRNA and protein expression of endogenous CLDN4. (C) Transient transfection of Twist1 was confirmed using western blot analysis. Down-regulation of CLDN4 was dose-dependent. (D) A schematic of pGL3.C4P1 bearing the full-length CLDN4 promoter and luciferase gene. Dose-dependent repression of CLDN4 promoter activity was seen as Twist1 expression increased by transient co-transfection (***$p < 0.001$).

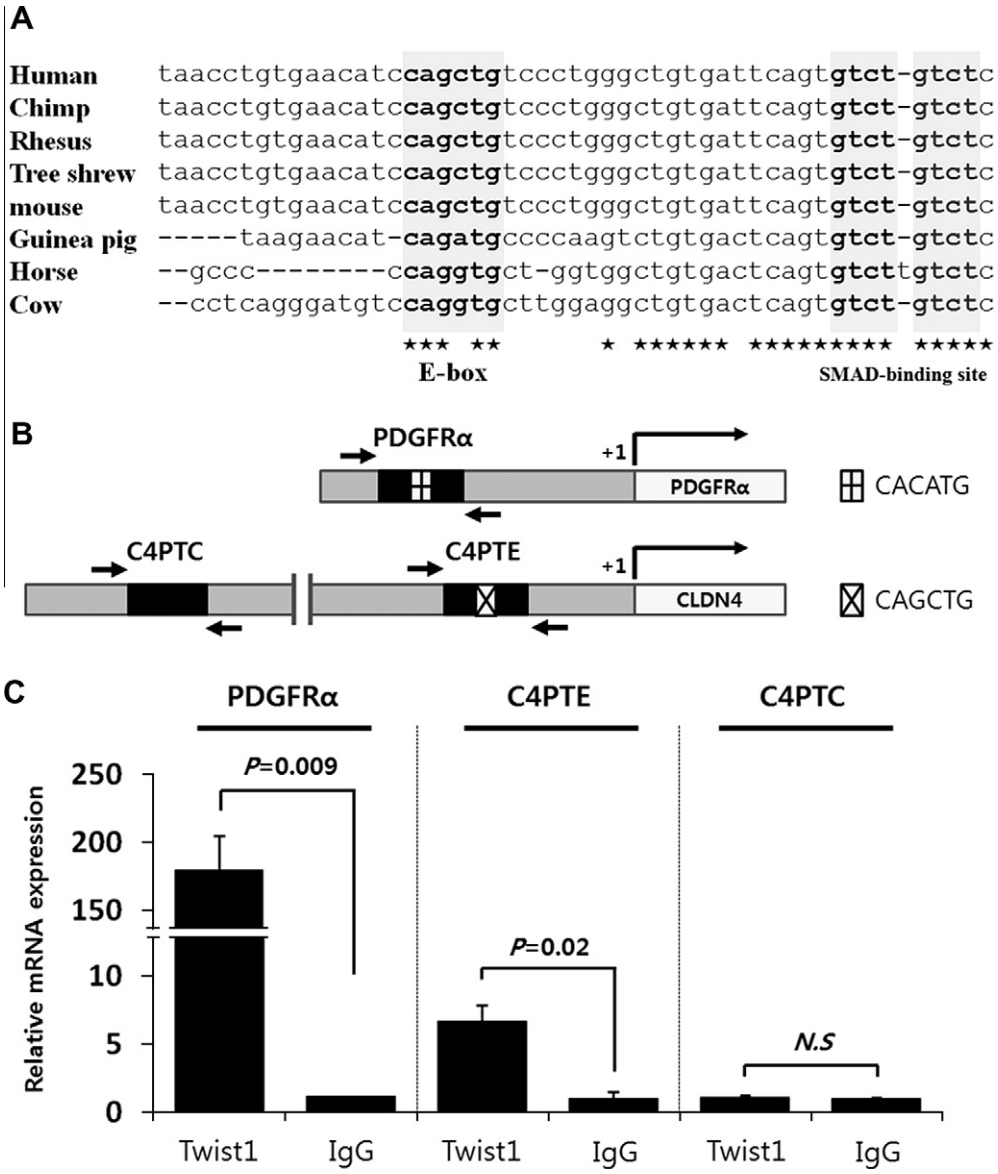

Fig. 2. Twist1 binds to its homological E-box site on the CLDN4 promoter. (A) Conserved motifs in the CLDN4 promoter. Alignment of an E-box sequence CAGNTG (in bold) for eight species. Dashes indicate an alignment gap. (B) Schematic illustrations of PDGFRα and CLDN4 promoter regions including each effective E-box site for Twist1. The arrows indicate the locations of the primers used for quantitative real-time RT-PCR. (C) After ChIP assays performed with anti-Twist1 antibody (Twist1) or control antibody (IgG), precipitants were analyzed with real-time PCR. In comparison to the IgG control, the PDGFRα or C4PTE region was specifically amplified by using each relevant primer set.

3.3. Binding to the E-box of Twist1 is sequence-specific for CLDN4 repression

Next, we examined a mutagenesis assay in order to validate our finding that the proximal E-box in the CLDN4 promoter could be a critical binding site for Twist1. The pGL3.C4P2 reporter vector encoding −322 to +42 bp of the sequence of the whole CLDN4 promoter was constructed and used as a control for comparison with E-box mutants. C4P2.E3M and C4P2.E5M mutants derived from pGL3.C4P2 were also constructed by site-specific mutagenesis of three- or five-nucleotide sequences in the E-box site (Fig. 3A). Then we confirmed that the luciferase activity was markedly decreased by co-transfection of pSG5.hTwist1.HA in pGL3.C4P2-introduced TE8 cells as it was in the pGL3.C4P1-introduced cells, indicating that CLDN4 promoter activity is suppressed by up-regulated Twist1 (Fig. 3B). We next used reporter assays with two mutated constructs to investigate whether binding of Twist1 to the CLDN4 promoter is specific to the E-box sequence. We observed a significant decrease in promoter activity after transient co-transfection with pSG5.hTwist1.HA into pGL3.C4P2-introduced TE8 cells, but when co-transfection was performed with C4P2.E3M or C4P2.E5M, no such decrease was seen (Fig. 3C). In addition, similar results in promoter activity were validated into another esophageal carcinoma TE10 cell line. These results indicate that mutation of the E-box sequence CAGCTG specifically abolished the inhibition of CLDN4 promoter activity induced by the co-transfection of Twist1 into esophageal cancer cells.

3.4. Twist1 and CLDN4 are inversely correlated in esophageal squamous cell carcinoma patients

To determine if the suppression of CLDN4 by Twist1 also occurs in esophageal cancer patient tissue, we examined Twist1 and CLDN4 expression in a larger number (n = 198) of formalin-fixed paraffin-embedded tissues from esophageal cancer patients by immunohistochemistry. Immunostaining data were categorized into four

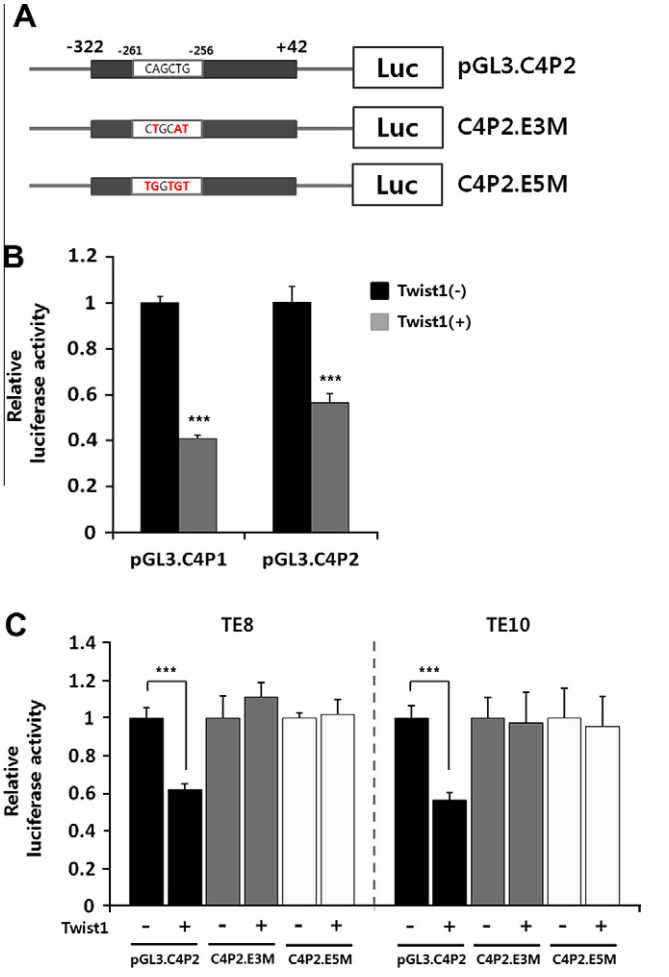

Fig. 3. Sequence-specific repression of CLDN4 by Twist1. (A) Schematic representation of a truncated construct of the CLDN4 promoter including the specific E-box site and mutant constructs including the substituted E-box sequence. (B) In TE8 cells, Twist1 co-transfected into TE8 cells effectively suppressed the luciferase activity of introduced pGL3.C4P2 (truncated) as well as pGL3.C4P1 (full-length) (***$p < 0.001$). (C) Sequence specificity of Twist1 binding to the E-box sequence in the CLDN4 promoter. In contrast with pGL3.C4P2, the promoter activities in both the C4P2.E3M and C4P2.E5M mutant constructs were not inhibited by up-regulated Twist1 in both TE8 and TE10 esophageal cancer cells (***$p < 0.001$).

groups according to the staining intensity of Twist1 (absent/low versus medium/high) or CLDN4 (low versus medium/high) (Fig. 4A). Representative cases showing strong CLDN4 membranous immunoreactivity without Twist1 expression in the nucleus and vice versa are shown in Fig. 4B. In addition, we found that CLDN4 immunoreactivity scores were markedly low in the patient group with highly up-regulated Twist1 ($n = 19$) and were conversely high in the Twist1-down-regulated group ($n = 179$), indicating that CLDN4 expression is significantly inversely related to Twist1 expression (Fig. 4C). Furthermore, survival rate varied greatly depending on status of Twist1 and CLDN4 expression. Specifically, the tumors with the Twist1-high/CLDN4-low expression had the worst clinical outcomes, whereas the tumors with the Twist1-low/CLDN4-high expression had the best prognoses (Fig. 4D). Both the Twist1-high/CLDN4-high and Twist1-low/CLDN4-low groups had intermediate prognoses. There was a tendency that the Twist1-high/CLDN4-high and Twist1-low/CLDN4-low tumors had worse prognosis than Twist1-low/CLDN4-high tumors, but no statistically significance. However, Twist1-high/CLDN4-low tumors have worse outcome than any of other remaining tumor subgroups with statistical significance ($P = 0.001$).

A

IHC score		CLDN4 (low vs medium/high)		Total
		low	high	
Twist1 (absent/low vs medium/high)	low	68	38	106
	high	74	18	92
Total		142	56	198

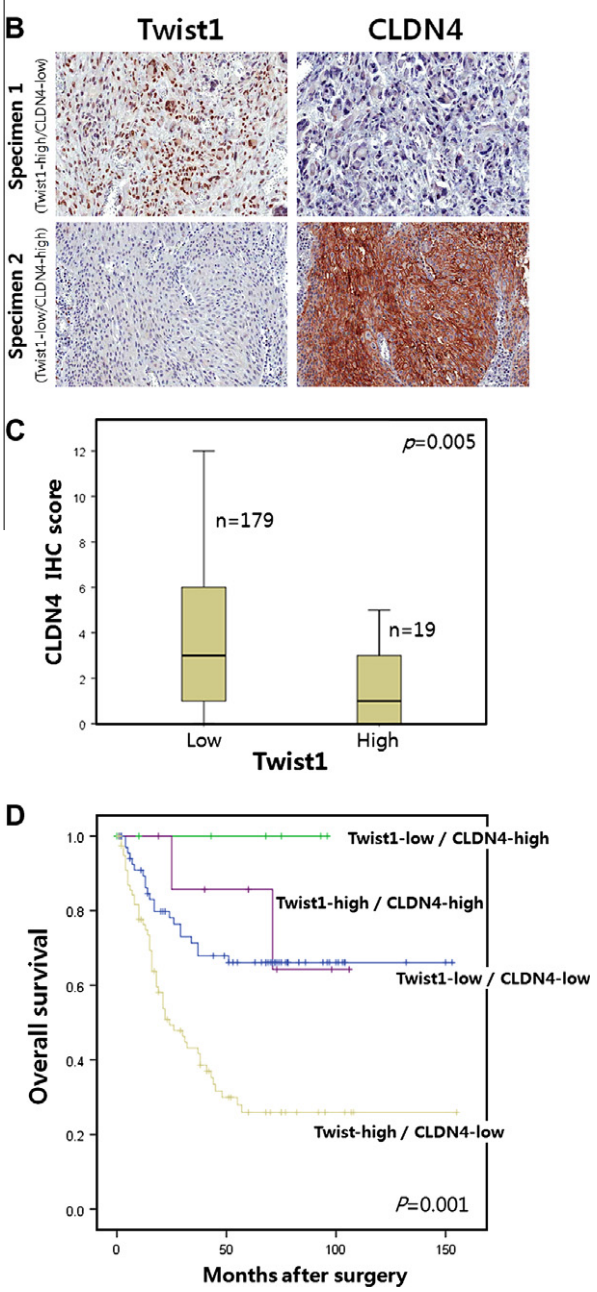

Fig. 4. Correlation between Twist1 and CLDN4 expression in esophageal cancer patient samples. (A) Results of immunohistochemical analysis of Twist1 and CLDN4 expression in 198 esophageal cancer samples. A significant inverse correlation between Twist1 and CLDN4 was determined by Fisher's exact test ($p = 0.012$). (B) Representative immunostaining results show inversely correlated patterns for Twist1 and CLDN4. (C) CLDN4 immunoreactivities in highly Twist1-overexpressed cases were significantly lower in all of the cases when compared to those absent/low for expression of Twist1 ($p = 0.005$). (D) Kaplan–Meier postoperative survival curves. Overall survival was defined based on the Twist1 and CLDN4 expression levels. Among all esophageal cancer patients, overall survival in Twist1-high/CLDN4-low cases was significantly worse than in other cases ($p = 0.001$).

Table 1
Multivariate Cox regression analysis of Twist1/CLDN4 and other covariates for ESCC patients' survival rate.

Predictors	Disease-free survival	
	HR (95% CI)	*P*-value
Age		
0–65	1.00	
65+	1.026 (0.605–1.740)	0.925
TNM Stage		
I	1.00	
II	2.227 (0.839–5.915)	0.108
III	4.074 (1.575–10.542)	0.004
IV	9.673 (3.501–26.725)	0.001
Chemotherapy		
Absent	1.00	
Positive	0.928 (0.574–1.501)	0.762
Radiation therapy		
Absent	1.00	
Positive	1.907 (1.216–2.989)	0.005
Twist1/CLDN4		
All other subgroups[a]	1.00	
Twist1-high/CLDN4-low	3.519 (2.177–5.687)	0.000

Abbreviations: HR, hazard ratio; CI, confidence interval.
[a] All other subgroups: Twist1-high/CLDN4-high + Twist1-low/CLDN4-low + -Twist1-low/CLDN4-high.

In addition, multivariate analysis identified Twist1-high/CLDN4-low expression pattern as a very strong adverse prognostic factor independent of stage, age, and chemo/radiation therapy ($P < 0.000$) (Table 1). And there are significant correlations between Twist1/CLDN4 expression patterns with several important clinicopathologic parameters such as stage, tumor invasion, tumor size, and tumor differentiation (Table 2). Taken together, our data demonstrate that suppression of CLDN4 expression by up-regulated Twist1 is closely related to a poor prognosis in esophageal cancer cases.

4. Discussion

We demonstrated that induction of Twist1 directly causes down-regulation of the junctional protein CLDN4 at the transcriptional level as well as the protein level and also suppresses the promoter activity of CLDN4 in a sequence-specific manner. To our knowledge, this is the first report to show down-regulation of CLDN4 via binding of Twist1 to a specific E-box site of the CLDN4 promoter in human esophageal carcinoma cells.

As we have shown previously [18] and in the present study, CLDN4 tends to be down-regulated in highly aggressive esophageal cancer and the reduction of CLDN4 leads to the resultant disruption of tight junctions that may be associated with EMT. Considering these observations, the repression of CLDN4 may be part of EMT development, which plays an important role in cancer metastasis. In addition, we previously demonstrated that Twist1 up-regulation induced EMT in esophageal cancer cell line such as TE1, 8, 10 and Twist1 overexpression was significantly correlated with EMT in patient tissue samples [21]. Based on our study, some CLDN4 repression may be attributable to Twist1 and other forms of repression may be induced by other EMT-inducing transcription factors such as Snail1, Snail2, ZEB1, and ZEB2. Snail1 has been shown to negatively modulate CLDN4 expression via its interaction with E-boxes in the CLDN4 promoter region in mouse epithelial cells [24]. However, it is not yet known which E-box site is involved in transcriptional control by Snail1. The involvement of other EMT transcriptional factors in CLDN4 repression remains to be explored.

Little has been shown previously about the repression of CLDN4 by Twist1. According to Vare and Soini [10], Twist expression was inversely associated with that of several claudins in testicular tumors. In their study, Twist-positive cases tended to be negative or weakly positive for CLDN1 ($p = 0.031$), CLDN3 ($p = 0.008$), CLDN4 ($p = 0.045$), and CLDN6 ($p = 0.039$). However, the study lacked a functional explanation for this observation. Mironchik et al. [25] reported that the induction of Twist1 led to the down-regulation of CLDN4 and CLDN7 in MCF-7, a breast cancer cell line, but this study also lacked an explanation for the mechanism of CLDN4 repression by Twist. Our results are highly consistent with these reports and furthermore, our study has revealed part of the mechanism involved in the relationship between these two proteins.

The gene-regulating activity of Twist1 as a transcription factor is initiated by binding of the protein to E-box sites of gene promoters as homo or heterodimer complexes. In this study, we focused on a specific, highly conserved E-box site (−261 to −256 bp) in the proximal region of the CLDN4 promoter. We also found a very well conserved Smad binding site (−244 to −237 bp) near the putative Twist1 binding E-box (Fig. 2A). Snail1, another EMT-inducing transcription factor like Twist1, also recognizes this same

Table 2
Analysis of Twist1/CLDN4 expression pattern in terms of their association with other clinicopathologic parameters of ESCC.

Stage		Twist-low/CLDN4-high	Twist-high/CLDN4-high	Twist-low/CLDN4-low	Twist-high/CLDN4-low	P
I	32	4 (42.9%)	7(75.0%)	10(11.4%)	11(11.5%)	0.001
II	68	4 (42.9%)	0(0.0%)	30(35.7%)	34(35.9%)	
III	73	1 (14.3%)	1(12.5%)	33(38.6%)	38(39.7%)	
IV	25	0 (0.0%)	1(12.5%)	12(14.3%)	12(12.8%)	
Depth of invasion (T)						
T1	40	5 (57.1%)	8 (87.5%)	11 (11.4%)	16 (16.7%)	0.001
T2	34	1 (14.3%)	0 (0.0%)	21 (24.3%)	12 (12.8%)	
T3	112	1 (14.3%)	1 (12.5%)	49 (57.1%)	61 (64.1%)	
T4	12	1 (14.3%)	0 (0.0%)	5 (5.7%)	6 (6.4%)	
Nodal metastasis						
Absent	79	5 (57.1%)	7 (75.0%)	28 (32.9%)	39 (41.0%)	0.091
Present	119	4 (42.9%)	2 (25.0%)	57 (67.1%)	56 (59.0%)	
Tumor size						
<5.0 cm	86	5 (57.1%)	8 (87.5%)	32 (37.1%)	41 (43.6%)	0.046
> = 5.0 cm	112	4 (42.9%)	1 (12.5%)	53 (62.9%)	54 (56.4%)	
Tumor grade						
W/D	32	5 (57.1%)	4 (37.5%)	10 (11.4%)	13 (14.1%)	0.018
M/D	131	4 (42.9%)	6 (62.5%)	55 (65.7%)	66 (69.2%)	
P/D	35	0 (0.0%)	0 (0.0%)	19 (22.9%)	16 (16.7%)	

kind of E-box sequence. Interestingly, Snail1 was reported to form a repressor complex with Smad3/4 and this complex promoted EMT [26]. In this case, the Snail1 binding E-box is also located close to the Smad binding site, like the conserved E-box site in the CLDN4 promoter. Based on this report, we can speculate that Twist1 may also form a repressor complex with the Smad protein, but this interaction remains to be verified. Our clinicopathologic study results suggested that the opposing expression of Twist1 and CLDN4, i.e., Twist1-high/CLDN4-low or Twist1-low/CLDN4-high, might be a key index for determining tumorigenic significance in esophageal squamous cell carcinoma.

The results of our clinicopathologic study using esophageal cancer patient tissues strongly imply the presence of a synergistic effect between Twist1-high and CLDN4-low on a patient's prognosis. That is, the combination of Twist1-high and CLDN4-low was significantly associated with an unfavorable clinical outcome compared to any other expression combination. Because Twist1 is involved in the regulation of EMT, the combination of Twist1-high and CLDN4-low may suggest that Twist1 is very active, thereby causing complete EMT; this may explain why the combination of Twist1-high and CLDN4-low was significantly associated with the most unfavorable clinical outcome. Consequently, our current data could serve as the basis for the suppression of esophageal cancer progression using Twist1 as a target and also suggest that decreased CLDN4 could serve as a prognostic index for esophageal cancer.

5. Disclosure statement

All the authors declared no conflict of interests.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.140.

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