remains unsettled, it appears to be associated with higher stage[2,5–7] and with worse prognosis than pure UC,[2,6–8] and it might be an indicator of poor response to chemotherapy and radiotherapy.[1,9] Therefore, it is important to detect the existence of squamous differentiation in bladder cancer. Keratinization and/or intercellular bridges are classic morphological features found in squamous differentiation in UC. However, recognition of non-keratinizing or very limited squamous differentiation is often difficult on morphological grounds alone. Some reports suggested that cytokeratin (CK)5/6, CK14, macrophage marker MAC387 and p63 were useful as immunohistochemical markers for squamous differentiation in UC. However, these markers could not distinguish precisely between squamous differentiation and UC.[3,10,11] There is an ongoing need for sensitive and specific immunohistochemical markers indicative of squamous differentiation in UC.

The desmosome is the major intercellular adhesive junction in squamous epithelia, and is composed of desmocollins (DSCs – DSC1, DSC2, and DSC3) and desmogleins (DSGs – DSG1, DSG2, and DSG3), which belong to the cadherin family of calcium-dependent cell adhesion molecules.[12] Previous immunohistochemical studies indicated that all non-cancerous squamous epithelia and 61–98% of systemic squamous cell carcinomas (SCCs) from the oral cavity, skin, oesophagus and lung were positive for several desmosomal isoforms.[13–16] Recently, it was reported that DSG3 could be a useful immunohistochemical marker to separate SCC from other histological subtypes of lung cancer, and had a sensitivity of 98% and specificity of 99% for lung SCC.[16] *DSC2* was identified as one of the genes that showed differential expression through cDNA microarray analysis of pure bladder SCC versus pure UC.[17] DSC2 is the most widespread and ubiquitous desmosome isoform.[18] Although it has been reported that DSC2 is expressed not only in skin and oesophageal SCC but also in gastric and colorectal adenocarcinoma,[19–22] there has been no immunohistochemical study of DSC2 in bladder cancer.

A combination of several markers might be helpful in the assessment of UC with squamous differentiation. Uroplakin III (UPIII), a transmembrane protein expressed by urothelial lining cells, has been shown to be a specific and relatively sensitive immunohistochemical marker of UC.[23,24] However, the significance of UPIII expression in UC with squamous differentiation remains unknown. Immunostaining of CK7 and CK20, which are intermediate filament proteins, has been shown to be a helpful diagnostic aid in the differentiation of tumours of primary unknown origin.[25,26] It has been reported that CK7 is often detected in systemic SCC,[25] and that CK20 is not detected in bladder SCC.[3,10] Therefore, CK7 and CK20 expression patterns in UC with squamous differentiation might be helpful in diagnosis.

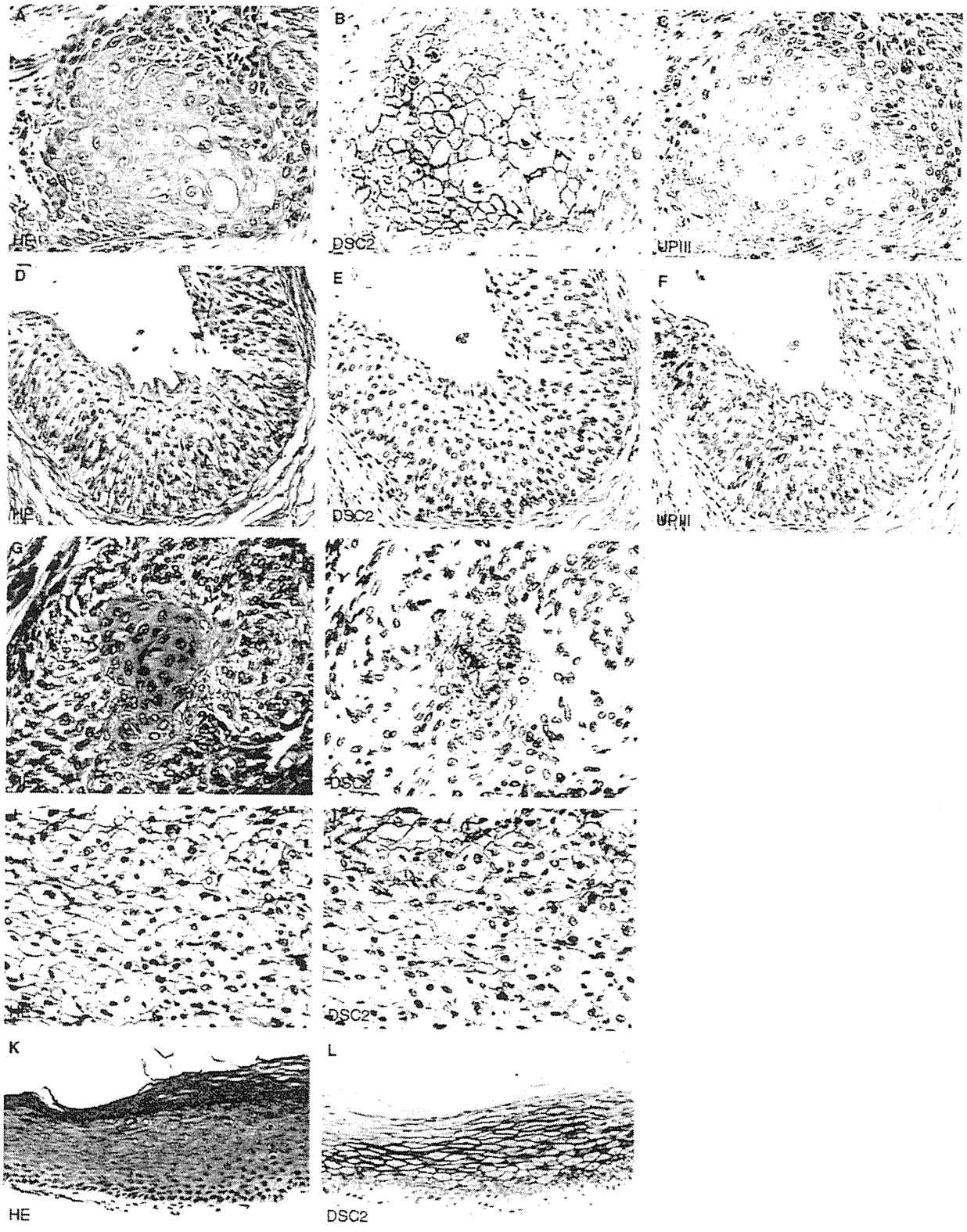
Several molecular markers appear to have some promising correlations with bladder cancer development. Among them, increased expression of epidermal growth factor receptor (EGFR) and p53 was reported to be significantly associated with high stage and poor prognosis.[27–31] Therefore, we examined the relationship between DSC2 staining and EGFR or p53 staining.

To our knowledge, the present study represents the first detailed analysis of DSC2 expression in bladder cancer. We performed immunohistochemical analysis of DSC2 in bladder cancer, and investigated the association between DSC2 expression and clinicopathological characteristics. In addition, to clarify the utility of DSC2 as a diagnostic marker, we compared the expression of DSC2 and that of various markers, including UPIII, CK7, and CK20, between 25 cases of UC with squamous differentiation and 85 cases of pure UC. We also evaluated the relationship between DSC2 expression and patient prognosis.

Materials and methods

TISSUE SAMPLES

Primary tumour samples were collected from 110 patients with bladder cancer (84 men and 26 women; age range, 33–86 years; mean, 68.1 years). Patients were treated at the Hiroshima University Hospital or affiliated hospitals. For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from these 110 patients, who had undergone radical cystectomy for bladder cancer, because radical cystectomy can give precise information on pathological stage and histology. Haematoxylin and eosin-stained sections from each case were re-evaluated histopathologically by two investigators (K.S. and T.H.), according to the World Health Organization classification.[32] Stage grouping and TNM pathological classification were carried out according to the International Union Against Cancer *TNM Classification of Malignant Tumors* (6th edition, 2002).[33] The 110 bladder cancer cases were histologically classified as pure UC in 85 cases and as UC with squamous differentiation in 25 cases. UC with squamous differentiation has some degree of squamous differentiation, which is defined by the presence of intercellular bridges or keratinization. In these 25 cases of UC, squamous differentiation occupied <50% of the tumour in 13 cases, 50–90% of the tumour in 10 cases, and >90% of



the tumour in two cases. Keratinization comprising 5–90% of the areas of squamous differentiation was present in all 25 (100%) cases of UC with squamous differentiation. Information on patient survival was available for 98 patients. In addition, for immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from 143 other patients who had undergone surgical excision for adenocarcinoma of the bladder ($n = 6$), oesophageal SCC ($n = 45$), lung SCC ($n = 27$), skin SCC ($n = 29$), or uterocervical SCC ($n = 36$), and from four patients with pure SCC of the bladder treated by transurethral resection of the bladder tumour. Because this was a retrospective study and written informed consent was not obtained from any patient, identifying information for all samples was removed before analysis for strict privacy protection. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government.

IMMUNOHISTOCHEMISTRY

Immunohistochemical analysis was performed with a Dako Envision+ Mouse Peroxidase Detection System (Dako Cytomation, Carpinteria, CA, USA). Antigen retrieval for DSC2 was performed by by proteinase K (Dako Cytomation) incubation for 5 min at 37°C in a humidified chamber. Antigen retrieval for UPIII, CK7 and CK20 was performed by microwave heating in citrate buffer (pH 6.0) for 30 min. After peroxidase activity had been blocked with 3% H_2O_2/methanol for 10 min, sections were incubated with normal goat serum (Dako Cytomation) for 20 min to block non-specific antibody binding sites. Sections were incubated with the following antibody dilutions: mouse monoclonal anti-DSC2, 1:50 (LifeSpan BioSciences, Seattle, WA, USA), mouse monoclonal anti-UPIII, 1:1 (Nichirei, Tokyo, Japan), mouse monoclonal anti-CK7 and anti-CK20, 1:50 (Dako Cytomation), mouse monoclonal anti-p53, 1:100 (Novocastra, Newcastle, UK), and mouse monoclonal anti-EGFR, 1:20 (Novocastra). This DSC2 antibody was the same as that used in our previous study.[20] Sections were incubated with primary antibody for 1 h at room temperature, and then with Envision+ anti-mouse peroxidase for 1 h. For colour reaction, sections were incubated with 3,3′-diaminobenzidine Substrate-Chromogen Solution (Dako Cytomation) for 10 min. Sections were counterstained with 0.1% haematoxylin. Because the aim of DSC2, UPIII and CK7/20 staining was to distinguish squamous differentiation from UC, the cut-off point for antibody reactivity necessary to define a result as positive was staining of any cells in surgically resected specimens. On the other hand, EGFR and p53 staining was considered to be positive if at least 10% of the tumour cells were stained, because the aim of this staining was to investigate the prognostic value.

CK7/20 PHENOTYPE OF UC

All 85 cases of UC, the 25 cases of UC with squamous differentiation and the 137 cases of systemic SCC were evaluated according to CK7 and CK20 staining patterns, and classified into four main groups: coexpression of CK7 and CK20 (CK7+/CK20+), no expression of CK7 or CK20 (CK7−/CK20−), expression of CK7 only (CK7+/CK20−), and expression of CK20 only (CK7−/CK20+).

WESTERN BLOT AND CELL LINES

For western blot analysis, cells were lysed as described previously.[34] Three cell lines derived from human oesophageal SCC and one cell line derived from human UC were used. Detailed information on western blot and cell lines is described in Data S1.

RNA INTERFERENCE (RNAI) AND OVEREXPRESSION OF DSC2 IN CELL GROWTH AND *IN-VITRO* INVASION ASSAYS

To knockdown the endogenous *DSC2*, RNAi was performed. Small interfering RNA (siRNA) oligonucleotides for *DSC2* and a negative control were purchased from Invitrogen (Carlsbad, CA, USA). Transfection was performed with Lipofectamine RNAiMAX (Invitrogen), as described previously.[35] For constitutive expression of DSC2, cDNA was amplified by PCR and subcloned into pcDNA 3.1 (Invitrogen). Transient transfection was carried out with the FuGENE6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA). To examine cell growth and invasiveness, respectively, 3-(4,5-

Figure 1. Immunohistochemical analysis of desmocollin (DSC)2 and uroplakin III (UPIII) in bladder cancer and non-neoplastic urothelium, using serial sections. A–C, Immunostaining of an area of squamous differentiation in urothelial carcinoma (UC) with squamous differentiation. DSC2 staining was detected, but UPIII staining was not detected. D–F, Immunostaining of pure UC. DSC2 staining was not detected, but UPIII staining was detected. G,H, In a case of UC with squamous differentiation, DSC2 expression was detected even in small foci of squamous differentiation. I,J, The tumour area adjacent to typical squamous differentiation was recognized as squamous differentiation by DSC2 staining. K,L, DSC2 expression was observed in keratinizing squamous metaplasia.

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)[36] and modified Boyden chamber assays were performed (see Data. S1).

STATISTICAL METHODS

Correlations between clinicopathological parameters and DSC2 staining, comparison of DSC2, UPIII, CK7, CK20, p53 and EGFR expression between squamous differentiation and UC, and CK7/20 expression patterns were analysed with Fisher's exact test or the chi-square test. Kaplan–Meier survival curves were constructed for patients positive or negative for DSC2, UPIII, CK7, and CK20, to compare survival between the groups. Differences in survival curves between groups were tested for statistical significance by the log-rank test.[37] The Cox proportional hazards multivariate model was used to examine the association of clinicopathological factors and the expression of DSC2, UPIII, CK7 and CK20 with survival. A P-value <0.05 was considered to be statistically significant.

Results

IMMUNOHISTOCHEMICAL STAINING IN BLADDER CANCER TISSUES

We performed immunostaining of DSC2 in the 110 cases of bladder cancer. DSC2 staining was detected in 24 of these 110 (22%) cases: in 24 of the 25 (96%) cases of UC with squamous differentiation, and in none of 85 (0%) cases of pure UC. Furthermore, DSC2 staining was detected in 24 of 25 (96%) areas of squamous differentiation and in none of 25 areas of UC in the 25 cases of UC with squamous differentiation. DSC2 staining was detected only in areas of squamous differentiation, and was observed in 5–90% of these areas. Two cases in which squamous differentiation occupied over 90% of the tumour, considered to have been similar to pure SCC, showed strong DSC2 expression in more than 80% of the areas of squamous differentiation. DSC2 expression was detected in all four additional cases of pure bladder SCC, and areas of DSC2 staining were observed in 60–90% of the SCCs. DSC2 expression was observed only in areas of squamous differentiation, and was not observed in UC (Figure 1A,B,D,E,G,H). DSC2 staining showed mostly the typical intercellular pattern seen in areas of squamous differentiation. In some cases, the tumour area adjacent to typical squamous differentiation partly expressed DSC2, and was recognized as squamous differentiation by DSC2 staining (Figure 1I,J). In non-neoplastic bladder mucosa, normal urothelial mucosa did not express DSC2, whereas keratinizing squamous metaplasia showed strong membranous staining (Figure 1K,L). Expression of DSC2 was not detected in stromal cells. Although it has been reported that gastric and colorectal adenocarcinomas are positive for DSC2,[19,20] DSC2 expression was not detected in six additional cases of bladder adenocarcinoma in the present study. We then analysed the relationship between DSC2 expression and clinicopathological characteristics (Table 1). DSC2 staining was detected significantly more frequently in stage III/IV (16/52 cases, 31%) than in stage 0/I/II (8/58 cases, 14%; $P = 0.0314$). There was no clear relationship between expression of DSC2 and age, sex, pT stage, pN stage, or pM stage.

Uroplakin III staining was detected in 41 of the 110 (37%) bladder cancer cases: in four of the 25 (16%) cases of UC with squamous differentiation, and in 37 of the 85 (44%) cases of pure UC. In the 25 cases of UC with squamous differentiation, UPIII staining was detected in none of the 25 areas of squamous differentiation, but was detected in four of 25 (16%) areas of UC. UPIII expression was observed in UC, but was not observed in areas of squamous differentiation (Figure 1A,C,D,F). UPIII expression was typically observed in umbrella cells and in focal cytoplasmic staining. There was no clear relationship between expression of UPIII and clinicopathological characteristics.

CK7 and CK20 staining was detected in 105 (95%) and 59 (54%) of the 110 bladder cancer cases: in 21 (84%) and five (20%) of the 25 cases of UC with squamous differentiation, and in 84 (99%) and 54 (64%) of the 85 cases of pure UC, respectively. In the 25 cases of UC with squamous differentiation, CK7 and CK20 staining was detected in 19 (76%) and three (12%) of 25 areas of squamous differentiation, and in 21 (84%) and five (20%) of 25 areas of UC, respectively. Cytoplasmic staining of CK7 and CK20 was observed in most of the cases with positive staining. There was no clear relationship between the expression of CK7 or CK20 and clinicopathological characteristics.

COMPARISON OF DSC2, UPIII, CK7 AND CK20 EXPRESSION BETWEEN THE AREAS OF SQUAMOUS DIFFERENTIATION IN UC WITH SQUAMOUS DIFFERENTIATION AND PURE UC

We compared the expression of DSC2 and UPIII in the area of squamous differentiation in the 25 cases of UC with squamous differentiation with that in the 85 cases of pure UC (Table 2). DSC2 expression was detected in 24 of 25 (96%) areas of squamous differentiation, but in none of 85 pure UCs. However, UPIII expression was

Table 1. Association between desmocollin (DSC)2 immunostaining and clinicopathological characteristics in bladder cancer tissues

	DSC2, no. (%)		
	Positive (n = 24)*	Negative (n = 86)	P-value†
Age (years)			
≤65	11 (27)	30	NS
>65	13 (18)	56	
Sex			
Male	16 (19)	68	NS
Female	8 (31)	18	
Histological classification			
Pure UC	0 (0)	85	<0.0001
UC with squamous differentiation	24 (96)	1	
pT stage			
0/1/2	10 (16)	52	NS
3/4	14 (29)	34	
pN stage			
0	17 (19)	74	NS
1/2	7 (37)	12	
pM stage			
0	22 (21)	84	NS
1	2 (50)	2	
Stage grouping			
0/I/II	8 (16)	50	0.0314
III/IV	16 (31)	36	

NS, Not significant; UC, urothelial carcinoma.
*The positive percentage is shown for each clinicopathological characteristic.
†Fisher's exact probability test.
Histology is according to the World Health Organization classification. Tumour staging and TNM pathological classification were carried out according to the TNM classification.

detected in 37 of the 85 (44%) UCs but in none of the 25 (0%) areas of squamous differentiation. When sensitivity, specificity and positive predictive value were calculated, DSC2 staining had a sensitivity of 96%, a specificity of 100% and a positive predictive value of 100% for the area of squamous differentiation, whereas UPIII staining had a sensitivity of 44%, a specificity of 100% and a positive predictive value of 100% for UC.

Table 2. Desmocollin (DSC)2, uroplakin III (UPIII), cytokeratin (CK)7 and CK20 expression in areas of squamous differentiation in 25 cases of urothelial carcinoma (UC) with squamous differentiation and 85 cases of pure UC

	Squamous differentiation (n = 25)*, no. (%)	UC (n = 85), no. (%)	P-value†
DSC2, no. (%)			
Positive	24 (96)	0 (0)	<0.0001
Negative	1	85	
UPIII			
Positive	0 (0)	37 (44)	<0.0001
Negative	25	48	
CK7, no. (%)			
Positive	19 (76)	84 (99)	0.0005
Negative	6	1	
CK20, no. (%)			
Positive	3 (12)	54 (64)	<0.0001
Negative	22	31	

*Squamous differentiation is the area of squamous differentiation in 25 cases of UC with squamous differentiation. The positive percentage is shown for each of the markers for squamous differentiation and UC.
†Fisher's exact probability test.

Next, we compared the expression of CK7 and CK20 between areas of squamous differentiation and UC. CK7 staining was detected significantly less frequently in areas of squamous differentiation (19/25 cases, 76%) than in areas of UC (84/85 cases, 99%; $P = 0.0005$). CK20 staining was also detected significantly less frequently in areas of squamous differentiation (3/25 cases, 12%) than in areas of UC (54/85 cases, 64%; $P < 0.0001$). After CK7/20 expression patterns were examined (Figure 2A), 16 (72%) areas of squamous differentiation were found to have a CK7+/CK20− pattern, six (24%) a CK7−/CK20− pattern, three (12%) a CK7+/CK20+ pattern, and 0 a CK7−/CK20+ pattern. Although the CK7+/CK20+ pattern was the predominant pattern in UC, the CK7+/CK20− pattern was the predominant pattern in squamous differentiation. The CK7+/CK20+ pattern was detected significantly less frequently in areas of squamous differentiation (3/25, 12%) than in UC (53/85 cases, 62%; $P < 0.0001$). The CK7+/CK20− pattern was detected significantly more frequently in areas of squamous differentiation (16/25 cases, 64%) than in UC (31/85 cases, 36%; $P = 0.0209$).

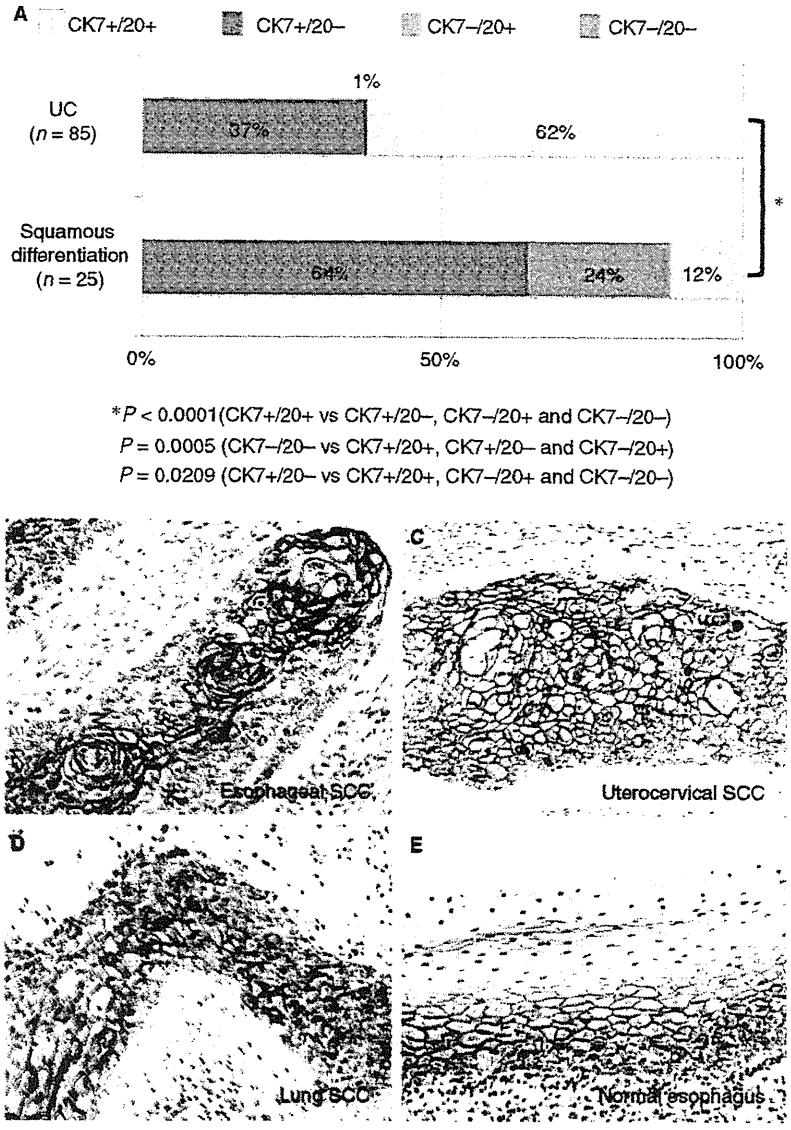


Figure 2. A, Summary of cytokeratin (CK)7/20 patterns compared between the area of squamous differentiation in 25 cases of urothelial carcinoma (UC) with squamous differentiation and 85 cases of pure UC. The CK7+/CK20– pattern was the predominant pattern in areas of squamous differentiation, and was detected more frequently in areas of squamous differentiation than in UC. *P*-values were determined by Fisher's exact test. B–E, Immunohistochemical staining of desmocollin (DSC)2 in systemic squamous cell carcinoma and non-neoplastic tissue of the oesophagus. Strong membranous and intercellular staining for DSC2 was observed.

IMMUNOHISTOCHEMICAL ANALYSES IN SYSTEMIC SCC

We performed immunostaining of DSC2 in systemic SCC. DSC2 staining was detected in all 45 (100%) oesophageal SCC cases, 23 of 27 (85%) lung SCC cases, 27 of 29 (93%) skin SCC cases, and 27 of 36 (75%) uterocervical SCC cases (Figure 2B–D). Histologically, well to moderately differentiated cases of SCC of the oesophagus, lung and skin and the keratinizing type of uterocervical SCC were all positive for DSC2 (Table 3). In non-neoplastic tissue of the oesophagus, DSC2 was expressed most strongly in the stratum spinosum, whereas the basal and granular layers were stained weakly (Figure 2E). These four types of SCC did not express UPIII. Next, the CK7/20 expression pattern was examined. The CK7+/CK20– pattern was predominant in uterocervical SCC (27/36 cases, 75%) and lung SCC (19/27 cases, 70%), similar to what was seen in areas of

squamous differentiation in UC with squamous differentiation (16/25 cases, 64%). The CK7−/CK20− pattern was predominant in SCC of the oesophagus (30/45 cases, 67%) and skin (19/29 cases, 66%).

RELATIONSHIP OF DSC2, UPIII, CK7 AND CK20 EXPRESSION AND CLINICOPATHOLOGICAL PARAMETERS WITH PATIENT PROGNOSIS

We also examined the relationship with patient prognosis of DSC2, UPIII, CK7 and CK20 expression and clinicopathological parameters. Univariate analysis revealed that DSC2 staining ($P = 0.0476$), UPIII staining ($P = 0.0493$), histological classification ($P = 0.0109$), stage grouping ($P < 0.0001$), pT stage ($P < 0.0001$), pN stage ($P < 0.0001$) and pM stage ($P = 0.001$) were all significant prognostic factors for survival in patients with bladder cancer (Figure 3B–E). However, CK7 staining, CK20 staining, age and sex did not correlate with survival. The Cox proportional hazards multivariate model was used to examine the association of DSC2 staining, UPIII staining, stage grouping, histological classification, pT stage, pN stage and pM stage with survival. Multivariate analysis revealed that stage grouping ($P = 0.0403$) was the only independent predictor of survival in patients with bladder cancer.

Table 3. Immunostaining of desmocollin (DSC)2 in systemic squamous cell carcinoma (SCC)

	DSC2	
	Positive, no. (%)	Negative, no.
Oesophageal SCC ($n = 45$)		
Well/moderately differentiated	36 (100)	0
Poorly differentiated	9 (100)	0
Lung SCC ($n = 27$)		
Well/moderately differentiated	16 (100)	0
Poorly differentiated	7 (64)	4
Skin SCC ($n = 29$)		
Well/moderately differentiated	21 (100)	0
Poorly differentiated	6 (75)	2
Uterocervical SCC ($n = 36$)		
Keratinizing	6 (100)	0
Non-keratinizing	21 (70)	9

EFFECTS OF DSC2 UP-REGULATION AND DOWN-REGULATION ON CELL GROWTH AND INVASIVE ACTIVITY

DSC2 expression was correlated with advanced stage of bladder cancer by immunohistochemistry. However, the biological significance of DSC2 in UC has not been studied; therefore, we studied the biological role of DSC2, using SCC and UC cell lines. Western blot analyses were performed in three oesophageal SCC cell lines (TE3, TE12, and TE13) and one UC cell line (T24). High-level DSC2 expression was noted in the well-differentiated SCC cell line TE3 and the moderately differentiated SCC cell line TE12, and low-level DSC2 expression was noted in the poorly differentiated SCC TE13 cell line as a band of approximately 99.9 kDa; the T24 cell line showed almost absent DSC2 expression (data not shown).

To investigate the possible antiproliferative effects of *DSC2* knockdown, we performed an MTT assay 8 days after siRNA transfection in the TE3 and TE12 cell lines. Cell viability was not significantly different between *DSC2* siRNA-transfected SCC cells and negative control siRNA-transfected SCC cells (data not shown). Next, to determine the possible role of DSC2 in invasiveness, a transwell invasion assay was performed in TE3 and TE12 cells. Invasion ability was not significantly different between *DSC2* knockdown SCC cells and negative control siRNA-transfected SCC cells (data not shown). Finally, to investigate the possible proliferative effect and invasiveness of DSC2 overexpression, we performed an MTT assay and transwell invasion assay using the UC cell line, T24. However, cell viability and invasion ability were not significantly different between T24 cells transfected with a *DSC2* expression vector (pcDNA-DSC2) and those transfected with a negative control vector (data not shown). These results indicate that DSC2 does not stimulate cell growth and invasion.

RELATIONSHIP BETWEEN EXPRESSION OF DSC2 AND EGFR

Increased expression of EGFR and p53 in UC was reported to be significantly associated with high stage and poor prognosis.[27–31] Therefore, we immunohistochemically examined the relationship between expression of DSC2 and expression of EGFR or p53. Although there was no clear relationship between staining of p53 and DSC2, EGFR staining was detected more frequently in DSC2-positive cases (16/24 cases, 67%) than in DSC2-negative cases (19/86 cases, 22%; $P < 0.0001$) (Table 4). Furthermore, EGFR staining was detected in 18 of 24 (75%) areas of squamous differentiation in

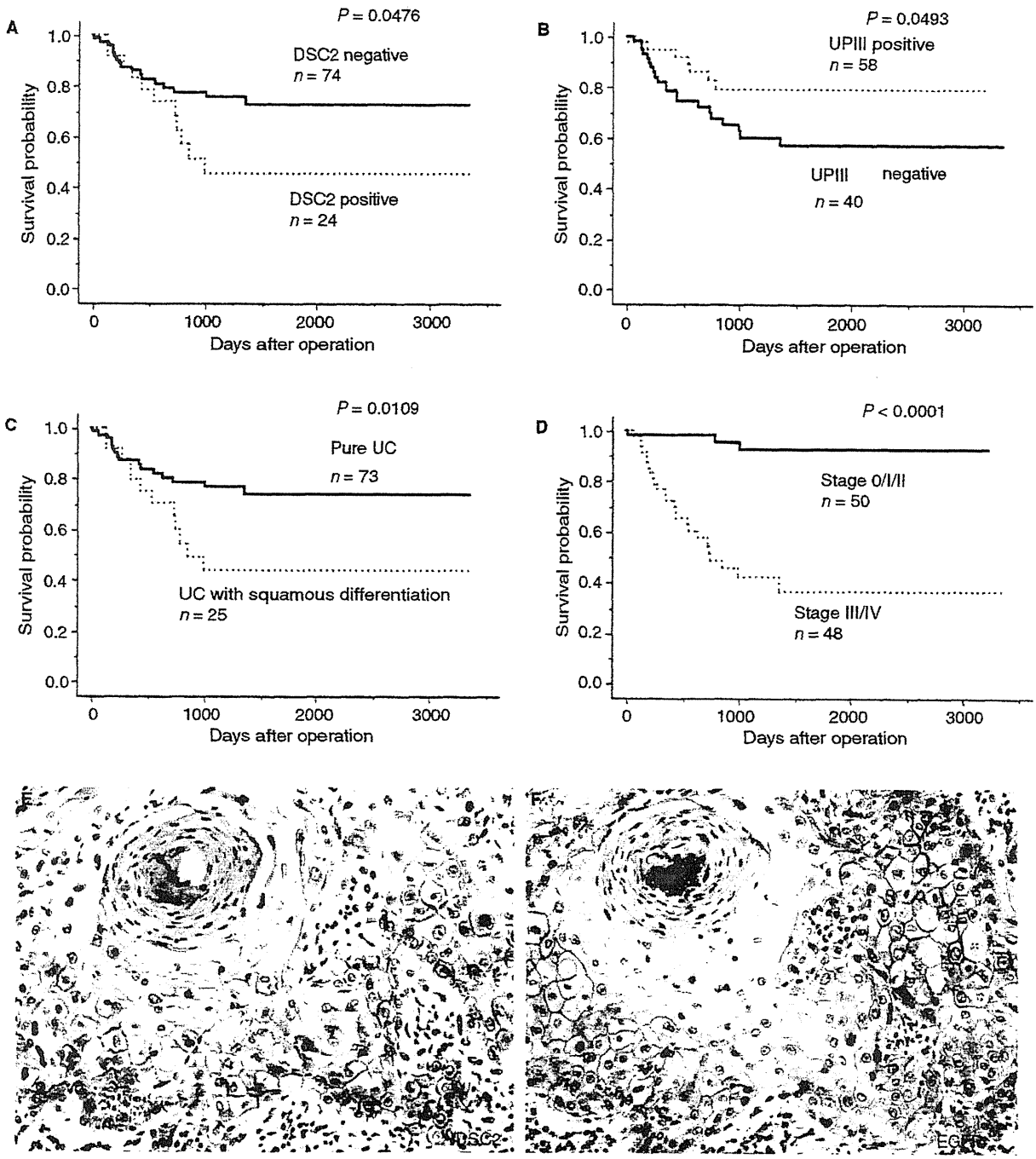


Figure 3. Prognostic values of desmocollin (DSC)2 staining (A), uroplakin III (UPIII) staining (B), histological classification (C) and stage grouping (D) in bladder cancer in 98 patients are shown by Kaplan–Meier curves. Survival of patients who were DSC2-positive or UPIII-negative, had urothelial carcinoma (UC) with squamous differentiation or had stage III/IV disease was significantly worse. *P*-values were determined by log-rank test. E,F, Immunohistochemical analysis of DSC2 and epidermal growth factor receptor (EGFR) in an area of squamous differentiation in UC with squamous differentiation, using serial sections. Squamous differentiation cells showing membranous staining of DSC2 also showed EGFR staining.

Table 4. Association between expression of desmocollin (DSC)2 and expression of epidermal growth factor receptor (EGFR) or p53

	DSC2, no. (%)		
	Positive (n = 24)*	Negative (n = 86)	P-value†
EGFR, no. (%)			
Positive	16 (67)	19 (22)	<0.0001
Negative	8	67	
p53, no. (%)			
Positive	10 (42)	28 (33)	NS
Negative	14	58	

NS, Not significant.
*The positive percentage is shown for each marker.
†Chi-square test.

DSC2-positive cases. Most of the areas of squamous differentiation positive for DSC2 also expressed EGFR (Figure 3E,F).

Discussion

Evidence of altered expression of desmosomal proteins in various human malignancies has been accumulating. In lung cancer, DSG3 and DSC3 were found to be useful markers for histological separation of SCC from other types of lung cancer.[16,38] DSG3 was overexpressed in head and neck SCC and correlated with stage, and inhibition of DSG3 in cell lines reduced cell growth and invasion.[15] However, decreased expression of DSCs and DSGs in skin and oral SCC correlated with poor differentiation and invasion or metastasis.[13,39] Desmosomal proteins are crucial components of adhesive junctions, and exhibit highly tissue-specific and isoform-specific patterns.

In the present study, DSC2 expression was correlated with UC with squamous differentiation in bladder cancer tissues. DSC2 staining was detected only in areas of squamous differentiation, and had a sensitivity of 96% and specificity of 100% for squamous differentiation in UC. Normal urothelium, UC and adenocarcinoma of the bladder did not express DSC2 in our study. In addition, systemic SCC showed high positivity (75–100%) for DSC2. Previous reports suggested that CK14 and the macrophage marker MAC387 might be helpful in the diagnosis of bladder SCC.[3,11] CK14 was reported to have high sensitivity and moderate specificity for pure bladder SCC, whereas MAC387 was reported to have high sensitivity and specificity for squamous differentiation in UC, but macrophages, monocytes, histiocytes and neutrophil leukocytes showed immunoreactivity for MAC387. CK5/6 and p63, which are markers for systemic SCC, are not useful for differentiation, because these markers are found in both UC and SCC. In our study, DSC2 appeared to be superior to previous markers, and intercellular bridge staining of DSC2 is the definition of squamous differentiation itself.

Squamous differentiation can be difficult to recognize in cases that are poorly differentiated. It has been reported that DSC2 membranous expression is reduced in accord with higher histological grade in oesophageal SCC.[22] In fact, DSC2 expression in our study was detected less frequently in the cases of poorly differentiated and non-keratinizing systemic SCCs than in those of well to moderately differentiated and keratinizing systemic SCCs. However, we found DSC2 expression in almost all cases of UC with squamous differentiation. Most areas of squamous differentiation in UC included several areas of keratinization, and DSC2 expression was detected even in small foci of squamous differentiation. DSC2 staining was very helpful in cases in which the diagnosis of squamous differentiation was difficult on morphological grounds alone. However, further studies are needed to clarify whether such cases can be identified by DSC2 staining prior to identification by morphological criteria, and larger prospective trials are needed to confirm these results.

Concerning the other three molecules studied, we confirmed that UPIII was not expressed in squamous differentiation, but did show high specificity in pure UC. UPIII expression was mutually exclusive of DCS2 expression in UC with squamous differentiation. Although several different cut-off points for positivity have been used in previous reports, expression of CK7 and CK20 in UC and systemic SCC in the present study agreed well with that reported in the literature.[24,25,40] We detected CK7 and CK20 staining less frequently in squamous differentiation than in UC, and the CK7+/20− pattern was predominant in squamous differentiation in UC. This pattern was different from that seen in UC, and was similar to that seen in lung and uterocervical SCC. Squamous differentiation in UC has a different expression pattern from that of UC for these molecules. With regard to changes in CK7/20 expression patterns, it can be speculated that alteration of DSC2 expression may affect the expression of CKs, because CKs are connected to desmosomal constitutive proteins. The expression patterns of these three molecules could not completely distinguish squamous differentiation from UC, but did provide additional diagnostic information.

Although pure bladder SCC appears to be more aggressive than pure UC after adjustment for stage and other prognostic factors,[4] the clinical significance of UC with squamous differentiation remains uncertain. In the present study, we showed that DSC2-positive cases correlated with higher stage than did cases of pure UC, and cases with DSC2 staining and UC with squamous differentiation had a worse prognosis by univariate analysis than those without; however, stage grouping was the only independent predictor of survival by multivariate analysis. These results are consistent with previous reports that UC with squamous differentiation tends to present at more advanced stages, and this higher stage is associated with a worse prognosis.[2,5–7] Although up-regulation and down-regulation of DSC2 did not affect cell growth and invasive activity, DSC2 staining was correlated with EGFR staining. EGFR functions as a tyrosine kinase that transduces signals controlling cell proliferation, and stimulates activation of activator protein-1 transcription factor with induction of matrix metalloproteinase activity in bladder cancer.[41,42] It has been reported that EGFR is detected in more than 90% of squamous lesions in the urinary bladder.[43,44] It was suggested that EGFR acquisition of DSC2-staining tumour cells is involved in cell proliferation and invasion. Further studies are needed to confirm the prognostic value of DSC2 and to clarify the relationship between DSC2 and EGFR.

In conclusion, we found that DSC2 staining showed high sensitivity and specificity for squamous differentiation in UC. Bladder cancers in which DSC2 staining was positive were at a more advanced stage than those not positive for DSC2. Our study emphasizes the value of immunohistochemistry in assessing squamous differentiation in UC. DSC2 appears to be a promising immunohistochemical marker of squamous differentiation in UC.

Acknowledgements

We thank Mr Shinichi Norimura for excellent technical assistance and advice. This work was carried out with the kind cooperation of the Research Centre for Molecular Medicine, Faculty of Medicine, Hiroshima University. We thank the Analysis Centre of Life Science, Hiroshima University, for the use of their facilities. This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Culture, Science, Sports and Technology of Japan, and in part by a Grant-in-Aid for the Third Comprehensive 10-Year Strategy for Cancer Control and for Cancer Research from the Ministry of Health, Labour and Welfare of Japan.

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Supporting Information

Additional Supporting information may be found in the online version of this article:

Data S1. Materials and methods.

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Original Paper

Pathobiology

Pathobiology 2011;78:277–284
DOI: 10.1159/000329588

Received: December 6, 2010
Accepted after revision: May 23, 2011
Published online: August 17, 2011

Identification of Transmembrane Protein in Prostate Cancer by the *Escherichia coli* Ampicillin Secretion Trap: Expression of CDON Is Involved in Tumor Cell Growth and Invasion

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Key Words
Prostate cancer · *Escherichia coli* ampicillin secretion trap · CDON

Abstract
Aims: Prostate cancer (PCa) is one of the most common malignancies worldwide. Genes expressed only in cancer tissue, and especially related to proteins located on the cell membrane, will be useful molecular markers for diagnosis and may also be good therapeutic targets. The aim of this study was to identify genes that encode transmembrane proteins present in PCa. ***Methods and Results:*** We generated *Escherichia coli* ampicillin secretion trap (CAST) libraries from 2 PCa cell lines and normal prostate tissues. By sequencing 3,264 colonies from CAST libraries, we identified 18 candidate genes that encode transmembrane proteins present in PCa. Quantitative RT-PCR analysis of these candidates revealed that *STEAP1*, *ADAM9* and *CDON* were expressed much more highly in PCa than in 15 kinds of normal tissues. Among the candidates, *CDON* encodes the CDO protein, which is an orphan cell surface receptor of the immunoglobulin superfamily. Additional quantitative RT-PCR revealed that 83% of PCa tissues showed *CDON* overexpression. Knockdown of *CDON* in DU145 cells induced 5-fluorouracil-induced apoptosis and inhibited invasion ability. ***Conclusion:*** These results suggest that *CDON* has a high potential as a therapeutic target for PCa.

Introduction

Internationally, prostate cancer (PCa) is the second most common cancer diagnosed among men, and is the sixth most common cause of cancer death among men [1]. Currently, the standard diagnostic marker for PCa is prostate-specific antigen (PSA) and the rapid incorporation of aggressive PSA testing has resulted in dramatically earlier identification of PCa [2]. Mortality from PCa is decreasing, which might be attributed to several factors, including increases in PSA screening and surgery, use of higher doses of radiotherapy, and earlier use of hormone therapy. However, the prognosis of advanced PCa

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still remains unsatisfactory [3]. Therefore, identification of new therapeutic targets for PCa is important.

PCa develops as a result of multiple genetic and epigenetic alterations [4]. Better knowledge of changes in gene expression that occur during carcinogenesis may lead to improvements in diagnosis, treatment and prevention. Genes encoding transmembrane/secretory proteins expressed specifically in cancers may be ideal biomarkers for cancer diagnosis. If the function of the gene product is involved in the neoplastic process, this gene may constitute a therapeutic target [5].

In the present study, to identify genes that encode transmembrane proteins present in PCa, we generated *Escherichia coli* ampicillin secretion trap (CAST) libraries from 2 PCa cell lines, LNCaP and DU145. CAST is a signal sequence trap method, developed by Ferguson et al. [6]. Signal peptides target secreted and transmembrane proteins to their appropriate subcellular location, and typically consist of 4–15 hydrophobic amino acids that are flanked by a basic NH2 terminus and a polar COOH terminus [7]. 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 is a survival-based signal sequence trap method that exploits the ability of mammalian signal sequences to confer ampicillin resistance to a mutant β-lactamase lacking the endogenous signal sequence [8]. We previously performed CAST analysis on 2 gastric cancer cell lines and identified several genes that encode transmembrane proteins present in gastric cancer [9]. However, CAST analysis of PCa has not been performed.

We report here the identification of genes that encode transmembrane proteins expressed in PCa. Among these, we focused on the *CDON* gene because this gene is frequently overexpressed in PCa, while its expression is narrowly restricted in normal tissues. *CDON*, which encodes CDO (cell adhesion molecule-related/down-regulated by oncogenes) protein, was cloned in 1997 from a cDNA library constructed from a rat embryo fibroblast cell line [10]. CDO protein is an orphan cell surface receptor of the immunoglobulin superfamily that is expressed on muscle precursor cells and developing muscles during mouse embryogenesis [10]. It positively regulates differentiation of myoblast cells [11], and is thought to be a strong candidate to mediate some of the effects of cell-cell contact that are important in myogenesis. However, the expression and function of *CDON* in human cancers including PCa have not been reported. In the present study, the effect of *CDON* knockdown was also investigated.

Materials and Methods

Cell Lines

LNCaP and DU145 PCa cell lines were purchased from American Type Culture Collection (Manassas, Va., USA). All cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (Whittaker, Walkersville, Md., USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

CAST Library Construction

pCAST was designed to contain the kanamycin resistance gene and the β-lactamase gene lacking the first 69 nucleotides encoding the endogenous signal peptide. EcoRI and BamHI sites were placed upstream of the mutant β-lactamase gene for directional cloning. CAST library construction was performed as described previously [6]. In brief, CAST cDNA libraries were generated from 2 μg of mRNA with a random primer containing a BamHI restriction site for reverse transcription (SuperScript Choice System; Invitrogen, Carlsbad, Calif., USA). The EcoRI-adapted cDNA was digested with BamHI, size fractionated, ligated into pCAST and plated onto Luris-Bertani/ampicillin. Individual colonies were picked and grown in 1.0 ml Luris-Bertani with kanamycin in a 96-well format. Plasmid DNA was sequenced in 96-well format using a primer located within the β-lactamase gene. The pCAST vector was kindly provided by Prof. Jonathan M. Graff (Department of Developmental Biology, University of Texas Southwestern Medical Center, Dallas, Tex., USA).

Tissue Samples

Subjects were 15 patients with PCa who were referred to the Department of Urology, Hiroshima University Hospital, Hiroshima, Japan. All PCa samples were obtained by radical prostatectomy and confirmed to be node negative by pathological examination. None of 15 patients with PCa received preoperative treatment. Samples were frozen immediately in liquid nitrogen and stored at −80°C until use. It was confirmed microscopically that the tumor specimens consisted mainly (>50%) of cancer cells. Tumor staging was according to the TNM classification system. Because written informed consent was not obtained, identifying information for all samples was removed before analysis in order to provide strict privacy protection. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government. Noncancerous samples of heart, lung, stomach, small intestine, colon, liver, pancreas, kidney, bone marrow, peripheral leukocytes, spleen, skeletal muscle, brain, spinal cord and prostate were purchased from Clontech (Palo Alto, Calif., USA).

Quantitative RT-PCR Analysis

Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, Calif., USA), and 1 μg of total RNA was converted to cDNA with a first-strand cDNA synthesis kit (Amersham Biosciences Corp., Piscataway, N.J., USA). Quantitation of CDON mRNA levels was done by real-time fluorescence detection as described previously [12]. The *CDON* primer sequences were 5′-TGG AAA TGA AGC CCC TCA GT-3′ and 5′-GAC GCT CTC CTC CGG CA-3′. PCR was performed with a SYBR Green PCR Core Reagents Kit (Applied Biosystems, Foster City, Calif., USA). Real-time detection of the emission intensity of SYBR green

Table 1. Properties of sequenced ampicillin-resistant colonies

	LNCaP	DU145	Normal prostate
Sequenced clones	1,344	960	960
Human named genes	234	228	224
Genes cloned in-frame upstream of the leaderless lactamase gene	96	120	72
Genes encoding secreted protein	6	8	7
Genes encoding transmembrane protein	39	55	37

bound to double-stranded DNA was performed with an ABI PRISM 7700 sequence detection system (Applied Biosystems) as described previously [13]. *ACTB*-specific PCR products were amplified from the same RNA samples and served as an internal control.

Evaluation of the Specificity of Gene Expression

To evaluate the specificity of expression of each gene, a specificity index was calculated as follows: first, we identified the normal tissue in which the target gene expression was highest among the 15 normal tissues analyzed by quantitative RT-PCR (the mRNA expression level in this tissue was denoted as A). We then identified PCa among the 9 PCa samples in which the target gene expression was highest by quantitative RT-PCR (the mRNA expression level in this tissue was denoted as B). The ratio B to A was defined as the specificity index. When the specificity index of the target gene was ≥ 10, the gene was considered to show a high specificity for PCa. When the specificity index of the target gene was <10 and ≥ 2, the gene was considered to show a low specificity for PCa. When the specificity index of the target gene was <2, the gene was considered to show no specificity for PCa.

RNA Interference

To knockdown the endogenous *CDON*, RNA interference was performed. Small interfering RNA (siRNA) oligonucleotides for CDON and a negative control were purchased from Invitrogen. Three independent oligonucleotides were used for *CDON* siRNA. The *CDON* siRNA1 sequence was 5′-UAU GGA GAG AGC UUG CAC CAG CUU G-3′. The *CDON* siRNA2 sequence was 5′-AAC AAC GGG AUA CUU AGG GAU GCC C-3′. The *CDON* siRNA3 sequence was 5′-UUA UGC AGC CAU GAG AUA CGA GUG G-3′. Transfection was performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. Briefly, 60 pmol siRNA and 10 μl Lipofectamine RNAiMAX were mixed in 1 ml 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. Forty-eight hours after transfection, cells were analyzed for all experiments.

Western Blot Analysis

For Western blot analysis, cells were lysed as described previously [14]. The lysates (40 μg) were solubilized in Laemmli sample buffer by boiling and then subjected to 10% SDS-polyacrylamide gel electrophoresis followed by electrotransfer onto a nitrocellulose filter. The filter was incubated with the primary antibody against CDO protein (rabbit polyclonal, dilution 1:500; Sigma Chemical, St. Louis, Mo., USA). Peroxidase-conjugated anti-rabbit IgG was used in the secondary reaction. Immunocomplexes were visualized with an ECL Western Blot Detection System (Amersham Biosciences). β-Actin antibody (Sigma Chemical) was also used as a loading control.

Cell Growth, Apoptosis and in vitro Invasion Assays

The cells were seeded at a density of 1,000 cells per well in 96-well plates. Cell growth was monitored after 1 and 2 days by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [15]. For apoptosis assay, cultured cells were treated for 48 h with 2 mM 5-fluorouracil (5-FU), and apoptosis was evaluated with an APOPercentage (Biocolor Ltd., Belfast, Ireland) in vitro apoptosis assay kit, according to the manufacturer's instructions. Modified Boyden chamber assays were performed to examine invasiveness as described previously [16]. Cells were plated at 10,000 cells per well in RPMI 1640 medium plus 1% serum in the upper chamber of a Transwell insert (8 μm pore diameter; Chemicon, Temecula, Calif., USA) coated with Matrigel. Medium containing 10% serum was added in the bottom chamber. After 1 and 2 days, cells in the upper chamber were removed by scraping and the cells remaining on the lower surface of the insert were stained with CyQuant GR dye to assess the number of cells.

Results

Generation of CAST Libraries

To identify genes that encode transmembrane proteins present in PCa, we generated CAST libraries from 2 PCa cell lines (LNCaP and DU145) and normal prostate tissues. We sequenced 1,344, 960 and 960 ampicillin-resistant colonies from each CAST library. We compared these sequences to those deposited in the public databases using BLAST (accessed at http://blast.ncbi.nlm.nih.gov/Blast.cgi), and evaluated the subcellular localization of the gene products using data from GeneCards (accessed at http://www.genecards.org/index.shtml). The properties of sequenced ampicillin-resistant colonies are shown in table 1. In total, we found that 39, 55 and 37

genes encoded transmembrane proteins, and 6, 8 and 7 genes encoded secreted proteins from LNCaP, DU145 and normal prostate CAST libraries, respectively. Because the purpose of this study was to identify genes that encode transmembrane proteins present in PCa, we focused on transmembrane proteins.

Identification of Genes Expressed More Highly in PCa than in Normal Tissues

To identify genes expressed specifically in PCa, we compared the gene list from each PCa cell line CAST library to the normal prostate CAST library. We only selected genes that were detected at least twice in each PCa cell line CAST library. In addition, genes were selected that were not found in the normal prostate CAST library. We obtained 6 candidates from LNCaP and 14 candidates from DU145. In total, 18 individual candidate genes were identified (table 2). To confirm that these candidates were PCa specific, quantitative RT-PCR was performed to measure the expression of these candidates in 9 PCa and 15 normal tissue samples. Representative results are shown in figure 1. Expression of the 18 candidate genes was not necessarily specific for PCa. However, several genes showed much higher expression in PCa than in normal tissues. We then focused on cancer specificity by calculating the specificity index for each gene (table 2). Of the 18 candidates, STEAP1 was found to show a high specificity for PCa, and 2 genes, *ADAM9* and *CDON*, were found to show a low specificity for PCa. The expression levels of the 18 genes in PCa tissue samples were also compared to those in normal prostate tissue samples. As shown in table 2, overexpression (tumor/normal prostate tissue ratio >2) was observed for all 18 genes. Among the 9 PCa cases, corresponding nonneoplastic prostate tissue samples were available for quantitative RT-PCR from 5 cases. The expression levels of the 18 genes in 5 samples of corresponding nonneoplastic prostate tissue were analyzed by quantitative RT-PCR. Frequent overexpression (tumor/nonneoplastic prostate tissue ratio >2) was found for all 18 genes (data not shown).

Overexpression of *STEAP1* and *ADAM9* in PCa has been reported [17, 18]; however, expression of *CDON* in human cancers including PCa has not been investigated. Therefore, expression of *CDON* was analyzed by quantitative RT-PCR in an additional 6 PCa samples and corresponding nonneoplastic prostate samples. We calculated the ratio of *CDON* mRNA expression levels between PCa tissue (T) and corresponding nonneoplastic prostate (N). T-to-N ratios >2 were considered to represent overexpression. Among 6 cases, 5 (83%) cases showed *CDON* overexpression.

Table 2. Summary of quantitative RT-PCR analysis of candidate genes specifically expressed in PCa

Gene name	Normal organ with highest expression		PCa (B)[1]	Specificity index (B/A)	PCa case No.[2]
	organ	mRNA expression level (A)			
High specificity					
STEAP1	pancreas	7.51	93.5[†]	12.5	9
Low specificity					
ADAM9	prostate	1.0	6.8	6.8	5
CDON	skeletal muscle	11.1	24.9	2.2	9
No specificity					
CTCL	pancreas	4.2	8.1	1.9	7
DSC2	heart	4.8	6.7	1.4	8
TNFRSF10D	pancreas	30.7	31.1	1.0	4
DNAJB14	heart	4.3	3.7	0.9	4
DLG1	pancreas	7.7	6.1	0.8	3
ADAM17	pancreas	3.8	2.8	0.7	2
SLC30A9	heart	32.0	20.2	0.6	5
PTP4A1	skeletal muscle	30.0	18.2	0.6	5
SGCE	heart	17.2	9.3	0.5	2
TMEM161B	pancreas	11.2	5.9	0.5	4
STX4	heart	12.2	4.3	0.4	4
LYSMD3	skeletal muscle	9.3	3.1	0.3	1
TM9SF3	pancreas	78.8	23.4	0.3	4
ITGB1	heart	23.4	6.7	0.3	2
TFRC	heart	42.5	10.4	0.2	4

The units are arbitrary. Target mRNA expression levels were standardized to 1.0 μg total RNA from normal prostate as 1.0.

[1] With highest expression mRNA expression level.

[2] With mRNA expression level 2-fold greater than normal prostate. [†] Arbitrary.

Effect of CDON Inhibition on Cell Growth and Invasive Activity

CDO protein is thought to be a strong candidate to mediate some of the effects of cell-cell contact that are important in myogenesis; however, the function of *CDON* in human cancers including PCa has not been reported. We performed Western blot analysis in LNCaP and DU145 cell lines. High CDO protein expression was found in DU145 cells as a band of approximately 139 kDa, and LNCaP cells had low or no CDO protein expression (fig. 2a). In CAST analysis, colonies containing *CDON* gene were detected twice in the DU145 CAST library, whereas no colonies with this gene were detected in the LNCaP CAST library, indicating that Western blot analysis of CDO protein was consistent with CAST anal-

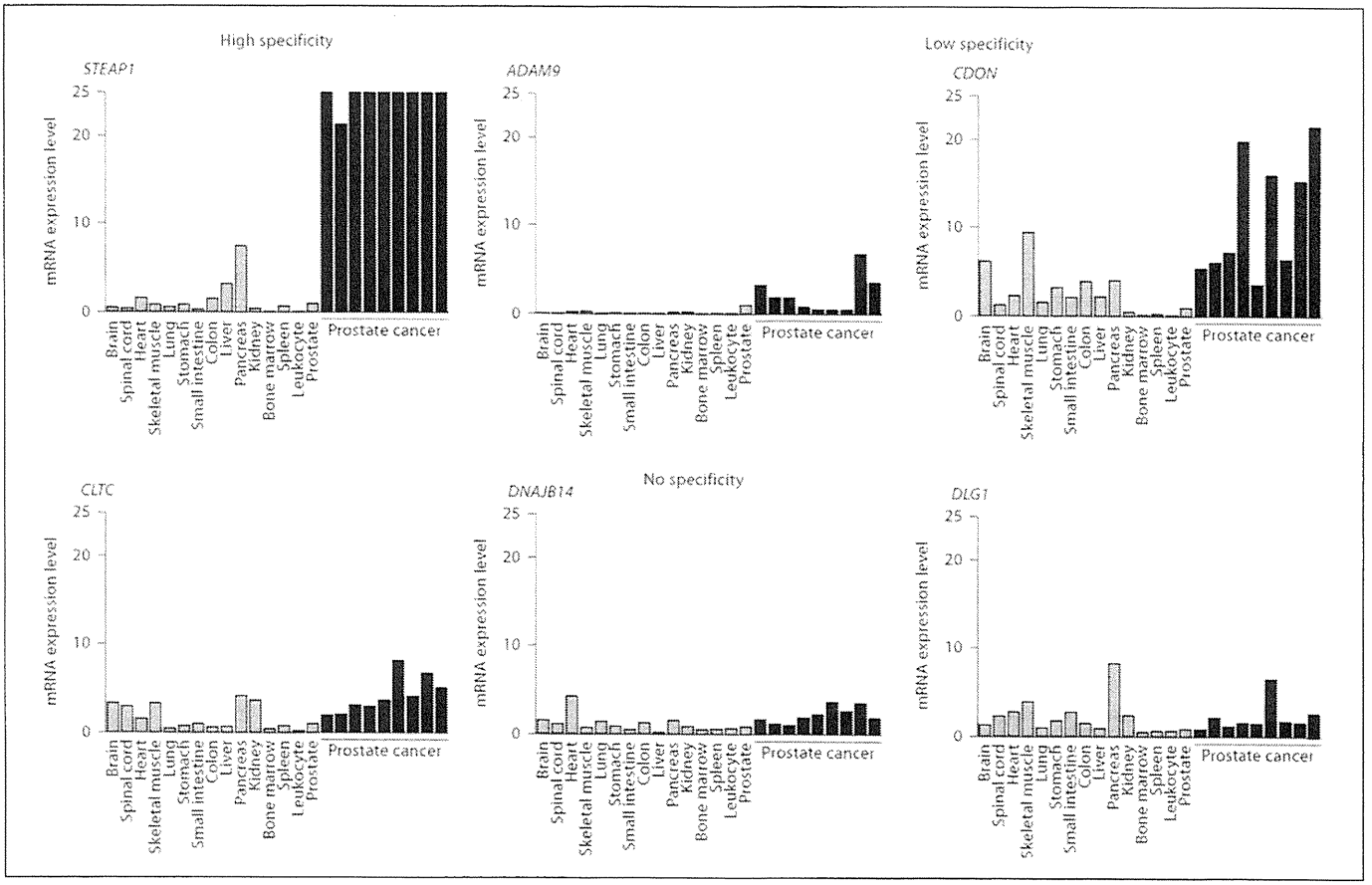

Fig. 1. Quantitative RT-PCR analysis of candidate genes that encode cell surface proteins in 15 normal tissues and 9 PCa samples. mRNA expression levels of *STEAP1*, *ADAM9* and *CDON* were much higher in PCa samples than in normal tissues. In contrast, mRNA expression levels of *CLTC*, *DNAJB14* and *DLG1* were not significantly different between PCa and normal tissues.

ysis. Next, we examined the transition of CDO protein expression by Western blot analysis of cell extracts of DU145 transfected with *CDON*-specific siRNAs. Three types of siRNAs (siRNA1–3) were transfected into DU145. The expression of CDO protein in DU145 was substantially suppressed by treatment with siRNA1, siRNA2 and siRNA3 (fig. 2b). We used siRNA1 in the following experiments to knock down the endogenous CDO protein. We also performed a time course analysis (from days 2 to 8) of *CDON* expression after siRNA transfection in DU145 cells. We confirmed suppression of *CDON* mRNA expression from days 2 to 8 after siRNA1 transfection by quantitative RT-PCR (data not shown).

To investigate the possible antiproliferative effect of *CDON* knockdown, we performed an MTT assay 8 days after siRNA transfection (fig. 2c). DU145 cells were selected for high CDO protein expression. *CDON* siRNA1-transfected DU145 cells showed significantly reduced viability relative to negative control siRNA-transfected DU145 cells. We performed the same assay using an LNCaP cell line that expressed low-level CDO protein. Reduced cell viability was not observed in siRNA1-transfected LNCaP cells compared with negative control siRNA-transfected LNCaP cells (fig. 2d). These results suggest that cell proliferation was activated or apoptosis was inhibited in *CDON* siRNA1-transfected DU145 cells. We examined the effect of *CDON* knockdown on the apoptotic susceptibility of DU145 cells to 5-FU. The frequency of apoptoitc cells in *CDON* siRNA1-transfected DU145 cells (mean, 71%) was significantly higher than that in negative control siRNA-transfected DU145 cells (mean 42%, $p = 0.0261$).

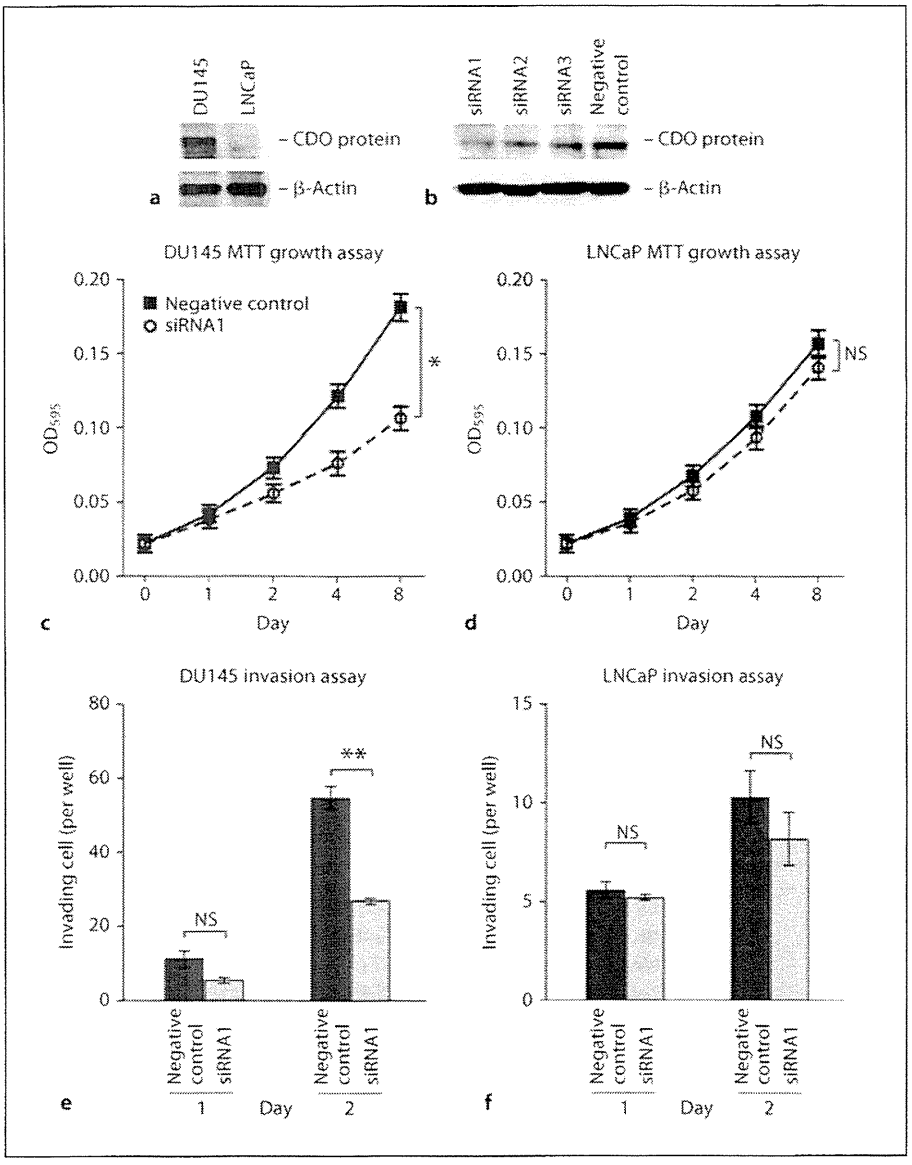

Fig. 2. CDO protein expression and functional analysis. Western blot analysis of CDO protein in PCa cell lines (**a**). Western blot analysis of CDO protein in DU145 cells transfected with the negative control siRNA and *CDON* siRNA (siRNA1–3) (**b**). Effect of *CDON* knockdown on cell growth of DU145 cells (**c**). Cell growth was assessed by an MTT assay at 1, 2, 4, and 8 days after seeding on 96-well plates in DU145 cells. Bars and error bars show means and SE of 3 different experiments. Effect of *CDON* knockdown on cell invasion of DU145 cells (**d**). After 1 and 2 days, invading cells were counted. Bars and error bars show means and SE of 3 different experiments. Cell growth was assessed by an MTT assay at 1, 2, 4 and 8 days after seeding on 96-well plates in LNCaP cells (**e**). Bars and error bars show means and SE of 3 different experiments. Effect of *CDON* knockdown on cell invasion of LNCaP cells (**f**). After 1 and 2 days, invading cells were counted. Bars and error bars show means and SE of 3 different experiments. NS = Not significant. * p = 0.0001; ** p = 0.0229.

Next, to determine the possible role of CDO protein in the invasiveness of PCa cells, we used a transwell invasion assay (fig. 2e). On day 2, although cell viability was not significantly different between *CDON* knockdown DU145 cells and negative control siRNA-transfected DU145 cells, the invasiveness of *CDON* knockdown DU145 cells was 50% less than that of the negative control siRNA-transfected DU145 cells. In contrast, invasion ability was not significantly different between *CDON* knockdown LNCaP cells and negative control siRNA-transfected LNCaP cells (fig. 2f). These results indicate that CDO protein stimulates invasion in PCa cells.

Discussion

We generated CAST libraries from 2 PCa cell lines, and identified several genes that encode transmembrane proteins present in PCa. Quantitative RT-PCR revealed that *STEAP1*, *ADAM9* and *CDON* were expressed much more highly in PCa than in 15 types of normal tissues. *STEAP1* showed the highest specificity for PCa. *STEAP1* encodes the STEAP-1 protein, which is a six-transmembrane cell surface protein. It has been reported that mouse monoclonal antibodies specific to STEAP-1 extracellular loops inhibit the growth of prostate and bladder tumor xenografts [17]. *ADAM9* showed the second highest spec-

ificity for PCa. ADAM proteins are zinc metalloproteases, which are involved in ectodomain shedding of growth factors, adhesion molecules, cytokines and receptors. ADAM9 has been shown to have proteolytic activity and its substrates include pro-HB-EGF, pro-EGF, the FGF receptor and so on [19, 20]. It has been reported that knockdown of *ADAM9* results in increased apoptosis and increased sensitivity to radiation in PCa cells [21]. These results indicate that both STEAP-1 and ADAM9 are therapeutic targets for PCa. In contrast, little is known about *CDON* expression in human cancers. In the present study, quantitative RT-PCR revealed that *CDON* was overexpressed in 83% of the PCa tissue samples, and knockdown of *CDON* in PCa cells induced 5-FU-induced apoptosis and inhibited invasion ability. These results suggest that *CDON* has the potential to constitute a therapeutic target for PCa.

The biological function of CDO protein is poorly understood. Previously, Kang et al. reported that CDO protein may have transformation suppressor function [10]. Expression of CDO protein was down-regulated in a rat embryo fibroblast cell line that was transformed by various oncogenes [10]. In addition, CDO protein levels were reduced when confluent cells were stimulated to re-enter the cell cycle. These results suggest that CDO protein may serve as a negative regulator of cell proliferation, transformation, and/or tumorigenesis. In the present study, knockdown of *CDON* in PCa cells induced 5-FU-induced apoptosis and inhibited invasion ability. Our present results are inconsistent with the previous reports, in which the function of CDO protein was investigated in rat embryo fibroblasts [10]. Here, we found that CDO protein in PCa cells may be different from fibroblasts.

On the other hand, it has been shown that the Sonic Hedgehog (SHh) directory interacts with CDO protein [22], and CDO protein functions to positively regulate SHh signaling in vivo and in vitro [23]. The Hh signaling pathway plays a central role during embryonic development and acts in stem cell renewal as well as tissue repair [24]. Hh ligands act via several components, including Patched and Smoothened transmembrane receptors, and lead to the activation of the Gli, a zinc-finger transcription factor. Several lines of evidence have indicated an association between Hh pathway activation and initiation or growth of several human cancers, including basal cell carcinomas, medulloblastomas and PCa [25, 26]. Therefore, it is possible that in addition to Patched, CDO protein also leads to the activation of the SHh pathway and participates in tumor cell growth in PCa.

Although *CDON* mRNA upregulation was observed in bulk PCa tissues by quantitative RT-PCR, expression and distribution of CDO protein in PCa tissues remains unclear. In this viewpoint, we performed immunohistochemical analysis in PCa tissue samples; however, obvious membranous staining was not found. The antibody against CDO protein used in the present study is not suitable for immunostaining because the antibody against CDO protein detected multiple bands on Western blots. Production of a specific antibody against CDO protein for immunostaining is required.

In summary, our present study yielded a list of genes that encode transmembrane proteins present in PCa. Our current data also provide information with respect to the expression of these genes throughout the body. We found that *CDON* is overexpressed in PCa, and *CDON* expression is narrowly restricted in normal tissues. Functional and immunohistochemical analysis will certify whether CDON may constitute a therapeutic target for PCa.

Acknowledgements

We thank Mr. Shinichi Norimura for his excellent technical assistance and advice. This work was carried out with the kind cooperation of the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University. We thank the Analysis Center of Life Science, Hiroshima University, for the use of their facilities. This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Culture, Science, Sports and Technology of Japan.

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Editorial

Pathobiology

Pathobiology 2011;78:293–294
DOI: 10.1159/000331226

Future Perspectives of Gastric Cancer Treatment – From Bench to Bedside

Cancer, a chronic proliferative disease with multiple genetic and epigenetic alterations, develops as a result of an accumulation of various endogenous and exogenous causes. Recent advances in cancer research have uncovered the molecular mechanisms of the development and progression of the disease. Multiple alterations during carcinogenesis are found in tumor suppressor genes, oncogenes, DNA repair genes, cell cycle regulators, cell adhesion molecules, growth factors/receptors, matrix metalloproteinases and other sites. Because of different but also common molecular bases, each cancer displays a different biological behavior and response to treatment. Recent focus has been on the presence of cancer stem cells in terms of chemoradiotherapy.

Amongst 10 million people diagnosed with cancer in the world, the most common cancers are those of the lung, breast and stomach. However, there are marked regional differences in the organs that are affected. In eastern Asia including Japan, Korea and China, cancers of the stomach, lung, liver and esophagus are of major concern, although cancers of the breast and colorectum are also increasing. Mortality due to gastric cancer, one of the most common cancers worldwide, is second to lung cancer. The highest rates of gastric cancer occur in eastern Asia, South America and Eastern Europe. Of particular note, more than half of the gastric cancer in the world occurs in Japan, China and Korea. Advances in diagnosis and treatment have enabled us to offer excellent long-term survival for early cancer, but the prognosis for advanced cancer still remains poor. The International Gastric Cancer Association, founded in 1995, contributes greatly to research into the carcinogenesis, diagnosis and treatment of gastric cancer; a congress on these issues was held recently, in 2011, in Seoul, Korea. Many scientific events on gastric cancer have been organized in these countries, such as the international symposium 'Future Perspectives of Gastrointestinal Cancer Treatment – From Bench to Bedside' at the occasion of the 20th Annual Meeting of the Japanese Society for Gastroenterological Carcinogenesis, the 17th Seoul International Cancer Symposium 'Gastric Cancer Update 2010', among others.

In this issue of *Pathobiology*, distinguished experts in Asia review current topics and future perspectives of gastric cancer treatment in molecular pathology, tissue engineering, surgery and chemotherapy. Woo et al. present the biological significance of STAT3 activation in gastric cancer. They analyzed a large number of gastric cancer specimens on tissue arrays immunohistochemically using several antibodies including anti-phospho-Tyr705-STAT3, an active form of STAT3. Nuclear STAT3 activation was an early event in carcinogenesis and significantly correlated with better prognosis, proliferation and HIF-1α activation in gastric cancer, suggesting that the nuclear pSTAT3 may serve as a valuable prognostic factor and therapeutic target in gastric carcinoma. Jang and Kim review the recent understanding of the molecular pathology of gastric cancer. Gastric cancer develops through multistep processes that begin with *Helicobacter pylori*-induced atrophic gastritis. Genetic and epigenetic

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