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 superfam-

ily. 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.

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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

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% CO₂ 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 μ g 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 Luria-Bertani/ampicillin. Individual colonies were picked and grown in 1.0 ml Luria-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 μ g 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'-UAA 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 μ l 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 μ g) 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 μ m 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					
<i>STEAP1</i>	pancreas	7.51	93.5 [†]	12.5	9
Low specificity					
<i>ADAM9</i>	prostate	1.0	6.8	6.8	5
<i>CDON</i>	skeletal muscle	11.1	24.9	2.2	9
No specificity					
<i>CTCL</i>	pancreas	4.2	8.1	1.9	7
<i>DSC2</i>	heart	4.8	6.7	1.4	8
<i>TNFRSF10D</i>	pancreas	30.7	31.1	1.0	4
<i>DNAJB14</i>	heart	4.3	3.7	0.9	4
<i>DLG1</i>	pancreas	7.7	6.1	0.8	3
<i>ADAM17</i>	pancreas	3.8	2.8	0.7	2
<i>SLC30A9</i>	heart	32.0	20.2	0.6	5
<i>PTP4A1</i>	skeletal muscle	30.0	18.2	0.6	5
<i>SGCE</i>	heart	17.2	9.3	0.5	2
<i>TMEM161B</i>	pancreas	11.2	5.9	0.5	4
<i>STX4</i>	heart	12.2	4.3	0.4	4
<i>LYSMD3</i>	skeletal muscle	9.3	3.1	0.3	1
<i>TM9SF3</i>	pancreas	78.8	23.4	0.3	4
<i>ITGB1</i>	heart	23.4	6.7	0.3	2
<i>TFRC</i>	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 se-

lected 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 apoptotic 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 knock-down 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 knock-down 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.

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