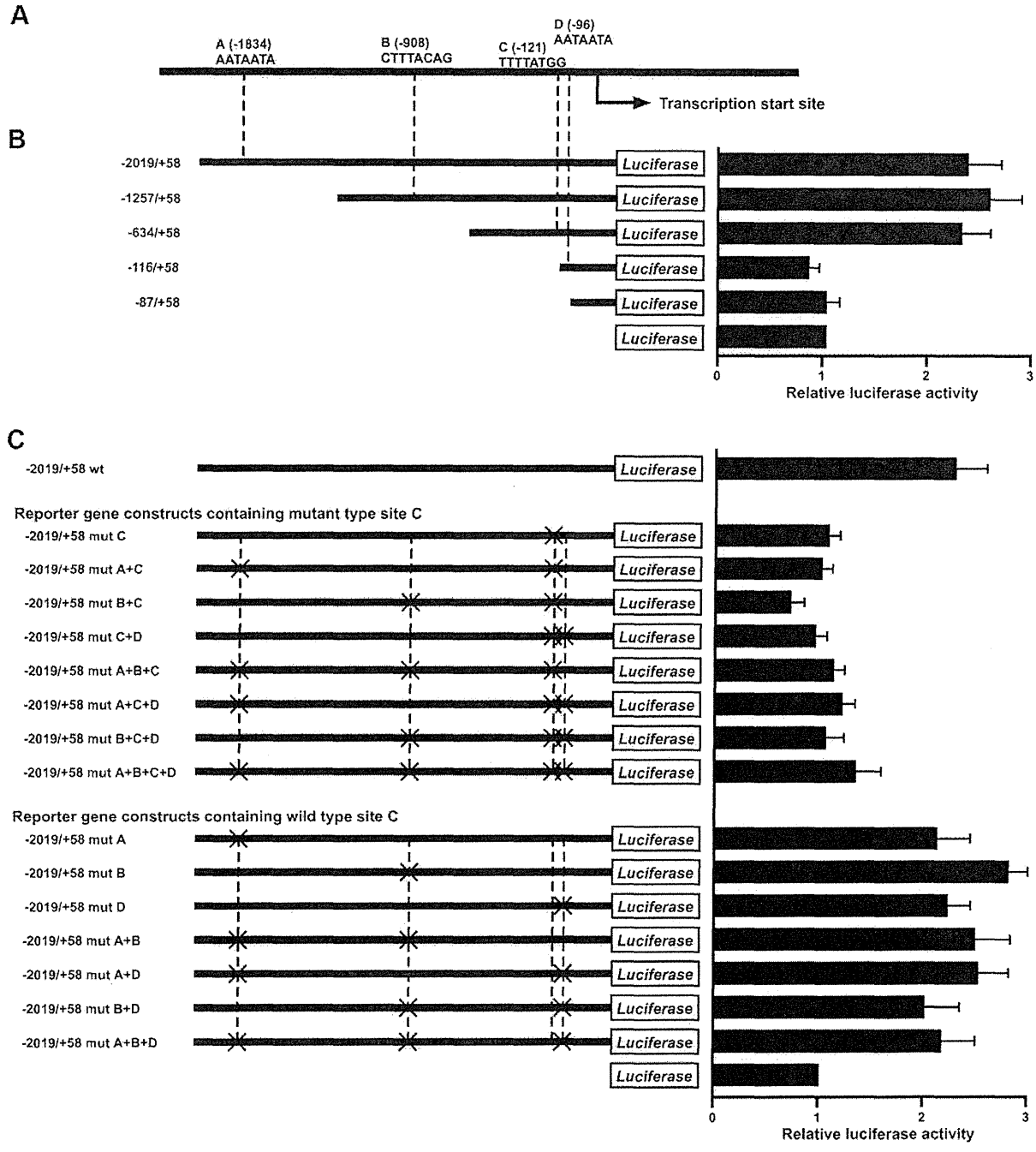


Figure 2. *REG4* promoter analysis. Localization of regulatory elements and CDX2 binding sites in the 5′-flanking region of the *REG4* gene. A: Schematic representation of the 5′-flanking region of the *REG4* gene. The location and sequence of 4 consensus CDX2-binding sites in the 5′-flanking region of *REG4* (i.e., sites A, B, C, and D) is indicated. B: Schematic representation of *REG4* reporter gene constructs. The *REG4* genomic DNA sequences present in the reporter gene vectors are indicated. Key sequences for *REG4* transcription reside between base pairs −634 and −116. Reporter assays with the series of *REG4* deletion constructs were performed in the CDX2-expressing GC cell line, HSC-39. The luciferase activity of the empty pGL4.10 basic vector was assigned a value of 1. The reporter assays were performed in triplicate, and mean and SD values of luciferase activity are shown. C: Localized mutations in the candidate CDX2-binding sites (i.e., sites A, B, C, and D) were introduced into the −2019/+58 construct, and the series of constructs generated is shown. The CDX2 candidate binding site designated as "C" plays critical roles in *REG4* transcription. Reporter assays were performed in CDX2-expressing GC cell line, HSC-39. The activity of the pGL4.10 basic vector was assigned a value of 1. Assays were performed in triplicate. Mean and SD luciferase activity values are shown.
doi:10.1371/journal.pone.0047545.g002

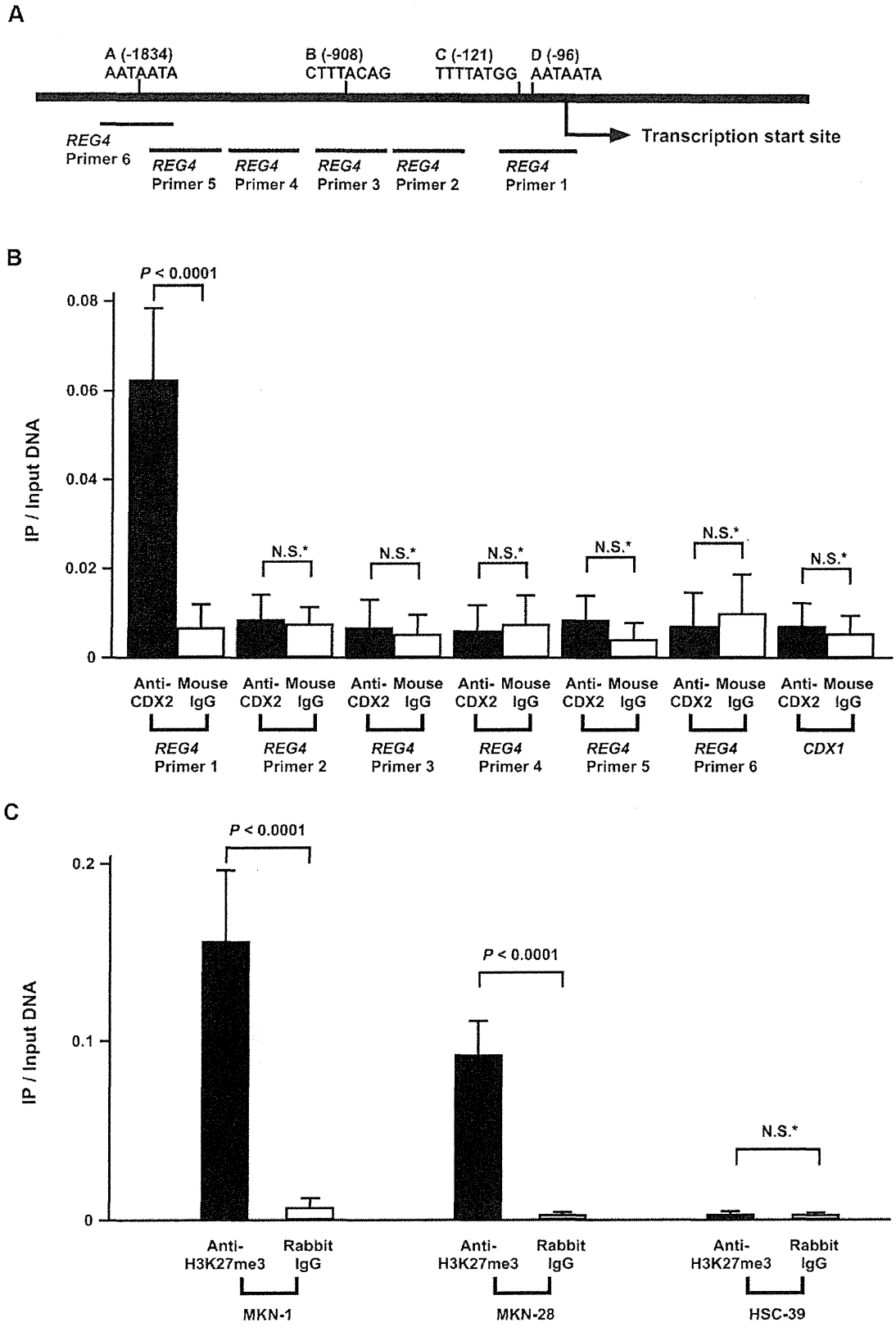

A
A (-1834)
AATAATA
B (-908)
CTTTACAG
C (-121)
TTTTATGG
D (-96)
AATAATA
Transcription start site
REG4
Primer 6
REG4
Primer 5
REG4
Primer 4
REG4
Primer 3
REG4
Primer 2
REG4
Primer 1
B
P < 0.0001
IP / Input DNA
0.08
0.06
0.04
0.02
0
N.S.*
Anti- CDX2
Mouse IgG
REG4 Primer 1
REG4 Primer 2
REG4 Primer 3
REG4 Primer 4
REG4 Primer 5
REG4 Primer 6
CDX1
C
P < 0.0001
IP / Input DNA
0.2
0.1
0
Anti- H3K27me3
Rabbit IgG
MKN-1
MKN-28
HSC-39

Figure 3. ChIP analysis of the 5′-flanking region of the *REG4* gene. A: Schematic representation of the 5′-flanking region of the *REG4* gene. The location of 4 consensus CDX2-binding sites in the 5′-flanking region of *REG4* (sites A, B, C, and D) and PCR primers (*REG4* Primer 1, 2, 3, 4, 5, and 6) are indicated. B: CDX2 binding to *REG4* promoter region shown by ChIP. Bulk (input) DNA was prepared as well as DNA isolated from ChIP with anti-CDX2 monoclonal antibody or mouse IgG. qPCRs were performed in triplicate for each sample primer set, and the mean and SD of the three experiments was calculated. C: ChIP analysis of H3K27me3 enrichment in the *REG4* gene promoter. ChIP enrichment was measured using qPCR. qPCRs were performed in triplicate for each sample primer set, and the mean and SD of the three experiments was calculated. *P* values were calculated using Student's t-test. * N.S. = not significant.
doi:10.1371/journal.pone.0047545.g003

transcriptional activity of reporter gene constructs containing 1.2 kb and 0.6 kb of *REG4* 5′-flanking sequence. As the effect of EGF or TGF-α on *REG4* transcription was not investigated in the present study, further investigation is needed to clarify the signalling mechanisms which induce regulation of *REG4* transcription.

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

Materials and Methods

Plasmids

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

Cell Lines, Retrovirus Infections, and Drug Treatment

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

Western Blot Analysis

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

qRT-PCR Analysis

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

RNAi

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

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

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

doi:10.1371/journal.pone.0047545.t001

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

Reporter Gene Assays

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

ChIP Assays

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

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

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

Acknowledgments

We thank Mr Shinichi Norimura for 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.

Author Contributions

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

References

1. Yasui W, Sentani K, Sakamoto N, Anami K, Naito Y, et al. (2011) Molecular pathology of gastric cancer: research and practice. Pathol Res Pract 207: 608–12.
2. Oue N, Hamai Y, Mitani Y, Matsumura S, Oshimo Y, et al. (2004) Gene expression profile of gastric carcinoma: identification of genes and tags potentially involved in invasion, metastasis, and carcinogenesis by serial analysis of gene expression. Cancer Res 64: 2397–405.
3. Aung PP, Oue N, Mitani Y, Nakayama H, Yoshida K, et al. (2006) Systematic search for gastric cancer-specific genes based on SAGE data: melanoma inhibitory activity and matrix metalloproteinase-10 are novel prognostic factors in patients with gastric cancer. Oncogene 25: 2546–57.
4. Hartupee JC, Zhang H, Bonaldo MF, Soares MB, Dieckgraefe BK (2001) Isolation and characterization of a cDNA encoding a novel member of the human regenerating protein family: Reg IV. Biochim Biophys Acta 1518: 287–93.
5. Bishnupuri KS, Luo Q, Murmu N, Houchen CW, Anant S, et al. (2006) Reg IV activates the epidermal growth factor receptor/Akt/AP-1 signaling pathway in colon adenocarcinomas. Gastroenterology 130: 137–49.
6. Legoffic A, Calvo E, Cano C, Folch-Puy E, Barthet M, et al. (2009) The reg4 gene, amplified in the early stages of pancreatic cancer development, is a promising therapeutic target. PLoS One 4: e7495.
7. Katsuno Y, Ehata S, Yashiro M, Yanagihara K, Hirakawa K, et al. (2012) Coordinated expression of REG4 and aldehyde dehydrogenase 1 regulating tumourigenic capacity of diffuse-type gastric carcinoma-initiating cells is inhibited by TGF-b. J Pathol, in press.
8. Mitani Y, Oue N, Matsumura S, Yoshida K, Noguchi T, et al. (2007) Reg IV is a serum biomarker for gastric cancer patients and predicts response to 5-fluorouracil-based chemotherapy. Oncogene 26: 4383–93.
9. Tatematsu M, Tsukamoto T, Inada K (2003) Stem cells and gastric cancer: role of gastric and intestinal mixed intestinal metaplasia. Cancer Sci 94: 135–41.
10. Motoshita J, Oue N, Nakayama H, Kuraoka K, Aung PP, et al. (2005) DNA methylation profiles of differentiated-type gastric carcinomas with distinct mucin phenotypes. Cancer Sci 96: 474–9.
11. Oue N, Mitani Y, Aung PP, Sakakura C, Takeshima Y, et al. (2005) Expression and localization of Reg IV in human neoplastic and non-neoplastic tissues: Reg IV expression is associated with intestinal and neuroendocrine differentiation in gastric adenocarcinoma. J Pathol 207: 185–98.
12. Takehara A, Eguchi H, Ohigashi H, Ishikawa O, Kasugai T, et al. (2006) Novel tumor marker REG4 detected in serum of patients with resectable pancreatic cancer and feasibility for antibody therapy targeting REG4. Cancer Sci 97: 1191–7.
13. Nakata K, Nagai E, Ohuchida K, Aishima S, Hayashi A, et al. (2009) REG4 is associated with carcinogenesis in the 'intestinal' pathway of intraductal papillary mucinous neoplasms. Mod Pathol 22: 460–8.
14. Oue N, Kuniyasu H, Noguchi T, Sentani K, Ito M, et al. (2007) Serum concentration of Reg IV in patients with colorectal cancer: overexpression and high serum levels of Reg IV are associated with liver metastasis. Oncology 72: 371–80.
15. Ohara S, Oue N, Matsubara A, Mita K, Hasegawa Y, et al. (2008) Reg IV is an independent prognostic factor for relapse in patients with clinically localized prostate cancer. Cancer Sci 99: 1570–7.
16. Sasahira T, Oue N, Kirita T, Luo Y, Bhawal UK, et al. (2008) Reg IV expression is associated with cell growth and prognosis of adenoid cystic carcinoma in the salivary gland. Histopathology 53: 667–75.
17. Hayashi T, Matsubara A, Ohara S, Mita K, Hasegawa Y, et al. (2009) Immunohistochemical analysis of Reg IV in urogenital organs: Frequent expression of Reg IV in prostate cancer and potential utility as serum tumor marker. Oncol Rep 21: 95–100.
18. Tamura H, Ohtsuka M, Washiro M, Kimura F, Shimizu H, et al. (2009) Reg IV expression and clinicopathologic features of gallbladder carcinoma. Hum Pathol 40: 1686–92.
19. Sentani K, Oue N, Noguchi T, Sakamoto N, Matsusaki K, et al. (2010) Immunostaining of gastric cancer with neuroendocrine differentiation: Reg IV-positive neuroendocrine cells are associated with gastrin, serotonin, pancreatic polypeptide and somatostatin. Pathol Int 60: 291–7.
20. Heiskala K, Arola J, Heiskala M, Andersson LC (2010) Expression of Reg IV and Hath1 in neuroendocrine neoplasms. Histol Histopathol 25: 63–72.
21. Wang F, Xu L, Guo C, Ke A, Hu G, et al. (2011) Identification of RegIV as a novel GLI1 target gene in human pancreatic cancer. PLoS One 6: e18434.
22. Nanakin A, Fukui H, Fujii S, Sekikawa A, Kanda N, et al. (2007) Expression of the REG IV gene in ulcerative colitis. Lab Invest 87: 304–14.
23. Silberg DG, Swain GP, Suh ER, Traber PG (2000) Cdx1 and cdx2 expression during intestinal development. Gastroenterology 119: 961–71.
24. Hinoi T, Lucas PC, Kuick R, Kuick R, Hanash S, et al. (2002) CDX2 regulates liver intestine-cadherin expression in normal and malignant colon epithelium and intestinal metaplasia. Gastroenterology 123: 1565–77.
25. Almeida R, Silva E, Santos-Silva F, Silberg DG, Wang J, et al. (2003) Expression of intestine-specific transcription factors, CDX1 and CDX2, in intestinal metaplasia and gastric carcinomas. J Pathol 199: 36–40.
26. Margalit Y, Yarus S, Shapira E, Gruenbaum Y, Fainsod A (1993) Isolation and characterization of target sequences of the chicken CdxA homeobox gene. Nucleic Acids Res 21: 4915–22.
27. Heinemeyer T, Wingender E, Reuter I, Hermjakob H, Kel AE, et al. (1998) Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL. Nucleic Acids Res 26: 362–7.
28. Jones PA, Baylin SB (2002) The fundamental role of epigenetic events in cancer. Nat Rev Genet 3: 415–428.
29. Mossman D, Scott RJ (2011) Long term transcriptional reactivation of epigenetically silenced genes in colorectal cancer cells requires DNA hypomethylation and histone acetylation. PLoS One 6: e23127.
30. Hinoi T, Tani M, Lucas PC, Caca K, Dunn RL, et al. (2001) Loss of CDX2 expression and microsatellite instability are prominent features of large cell minimally differentiated carcinomas of the colon. Am J Pathol 159: 2239–48.
31. Hinoi T, Gesina G, Akyol A, Kuick R, Hanash S, et al. (2005) CDX2-regulated expression of iron transport protein hephaestin in intestinal and colonic epithelium. Gastroenterology 128: 946–61.
32. Takakura Y, Hinoi T, Oue N, Sasada T, Kawaguchi Y, et al. (2010) CDX2 regulates multidrug resistance 1 gene expression in malignant intestinal epithelium. Cancer Res 70: 6767–78.
33. Anami K, Oue N, Noguchi T, Sakamoto N, Sentani K, et al. (2010) Search for transmembrane protein in gastric cancer by the Escherichia coli ampicillin secretion trap: expression of DSC2 in gastric cancer with intestinal phenotype. J Pathol 221: 275–84.
34. Pastan I, Gottesman MM (1991) Multidrug resistance. Annu Rev Med 42: 277–86.
35. Tajima Y, Shimoda T, Nakanishi Y, Yokoyama N, Tanaka T, et al. (2003) Association of gastric and intestinal phenotypic marker expression of gastric carcinomas with tumor thymidylate synthase expression and response to postoperative chemotherapy with 5-fluorouracil. J Cancer Res Clin Oncol 129: 683–90.
36. Motoshita J, Nakayama H, Taniyama K, Matsusaki K, Yasui W (2006) Molecular characteristics of differentiated-type gastric carcinoma with distinct mucin phenotype: LI-cadherin is associated with intestinal phenotype. Pathol Int 56: 200–5.
37. Grignani F, Kinsella T, Mencarelli A, Valtieri M, Riganelli D, et al. (1998) High-efficiency gene transfer and selection of human hematopoietic progenitor cells with a hybrid EBV/retroviral vector expressing the green fluorescence protein. Cancer Res 58: 14–19.
38. Ochiai A, Yasui W, Tahara E (1985) Growth-promoting effect of gastrin on human gastric carcinoma cell line TMK-1. Jpn J Cancer Res 76: 1064–71.
39. Yanagihara K, Seyama T, Tsumuraya M, Kamada N, Yokoro K (1991) Establishment and characterization of human signet ring cell gastric carcinoma cell lines with amplification of the c-myc oncogene. Cancer Res 51: 381–6.
40. Yanagihara K, Tanaka H, Takigahira M, Ino Y, Yamaguchi Y, et al. (2004) Establishment of two cell lines from human gastric scirrhous carcinoma that possess the potential to metastasize spontaneously in nude mice. Cancer Sci 95: 575–82.
41. Hoji H (1977) Establishiment of cultured cell lines of human stomach cancer – origin and their morphological characteristics. Niigata Igakukai Zasshi 91: 737–752.
42. Motoyama T, Hoji H, Watanabe H (1986) Comparison of seven cell lines derived from human gastric carcinomas. Acta Pathol. Jpn. 36: 65–83.
43. Sekiguchi M, Sakakibara K, Fuji G (1978) Establishment of cultured cell lines derived from a human gastric carcinoma. Jpn. J. Exp. Med. 48: 61–68.
44. Yasui W, Ayhan A, Kitadai Y, Nishimura K, Yokozaki H, et al. (1993) Increased expression of p34cdc2 and its kinase activity in human gastric and colonic carcinomas. Int J Cancer 53: 36–41.
45. Gibson UE, Heid CA, Williams PM (1996) A novel method for real time quantitative RT-PCR. Genome Res 6: 995–1001.
46. Kondo T, Oue N, Yoshida K, Mitani Y, Naka K, et al. (2004) Expression of POT1 is associated with tumor stage and telomere length in gastric carcinoma. Cancer Res 64: 523–9.

Original Paper

Pathobiology

Pathobiology 2013;80:60–69
DOI: 10.1159/000341396

Received: March 6, 2012
Accepted after revision: June 18, 2012
Published online: August 29, 2012

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

Tetsutaro Hayashi[a, b] Kazuhiro Sentani[a] Naohide Oue[a] Shinya Ohara[b] Jun Teishima[b] Katsuhiro Anami[a] Naoya Sakamoto[a] Akio Matsubara[b] Wataru Yasui[a]

Departments of [a]Molecular Pathology and [b]Urology, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, Japan

Key Words
CAST · NBL1 · Prostate cancer

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

Introduction

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

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1015–2008/13/0802–0060$38.00/0

Accessible online at:
www.karger.com/pat

Dr. Wataru Yasui
Department of Molecular Pathology
Hiroshima University Graduate School of Biomedical Sciences
1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551 (Japan)
Tel. +81 82 257 5145, E-Mail wyasui@hiroshima-u.ac.jp

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

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

Materials and Methods

CAST Library Construction

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

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

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

Tissue Samples

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

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

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

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

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

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

Quantitative RT-PCR and Western Blot Analysis

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

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

Evaluation of Specificity of Gene Expression

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

Immunohistochemistry

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

RNA Interference and Cell Growth and in vitro Invasion Assays

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

Statistical Method

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

Results

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

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

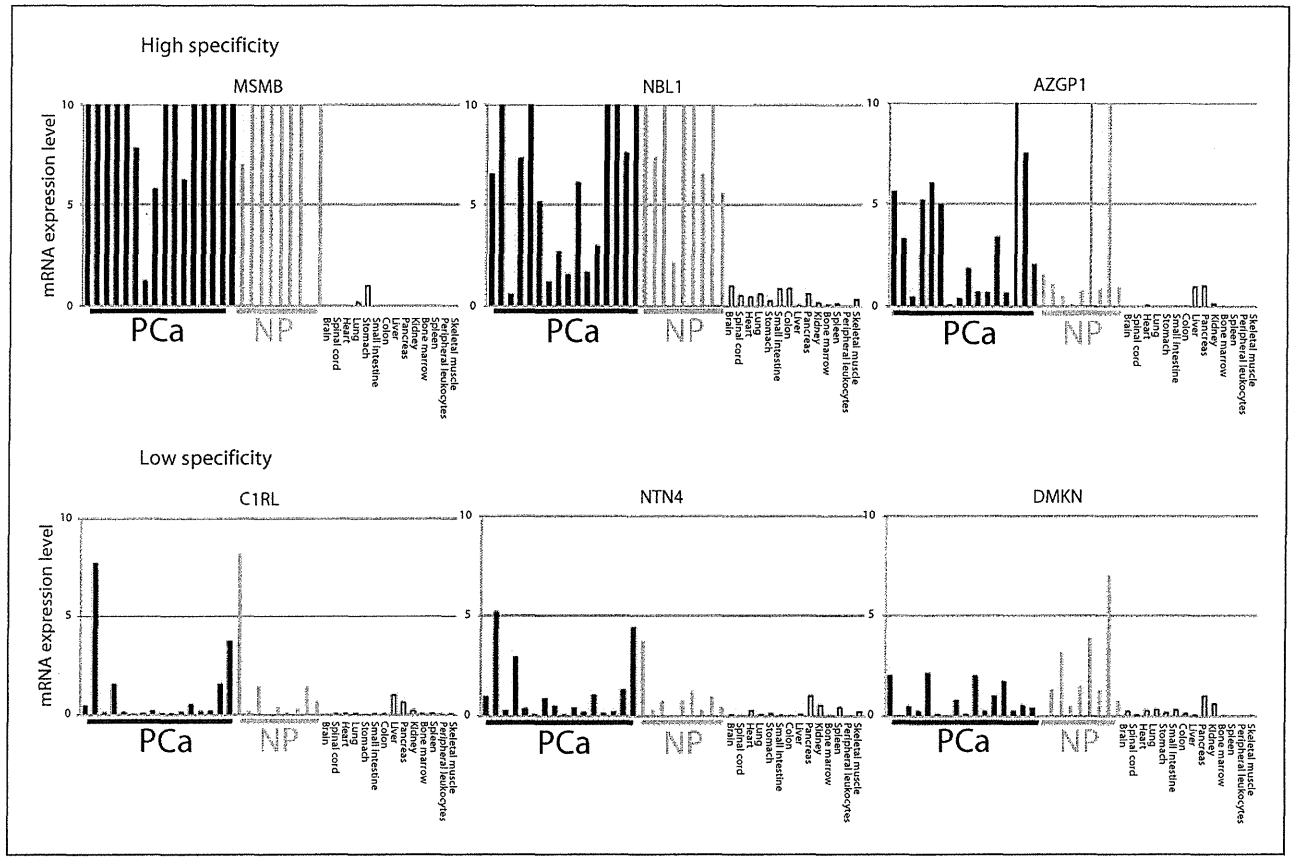


Fig. 1. Quantitative RT-PCR analysis of candidate genes in 16 PCa samples, 9 NP samples, and 14 kinds of normal tissues. MSMB, NBL1 and AZGP1 were found to show high specificity for the prostate. C1RL, NTN4 and DMKN were found to show low specificity for the prostate.

PSA is also specific for prostate and not specific for PCa, we examined these prostate-specific genes. Of the 15 candidates, 3 genes, *MSMB*, *NBL1* and *AZGP1*, were found to show high specificity for the prostate, and 3 genes, *C1RL*, *NTN4* and *DMKN*, were found to show low specificity for the prostate. Of the 3 genes showing high specificity, MSMB (microseminoprotein-β) and AZGP1 (zinc-α_2-glycoprotein) have already been studied in PCa, and their utility as serum tumor marker and therapeutic target have been reported [24–27]. Therefore, we focused on *NBL1*. *NBL1* has been reported as one of the genes whose expression is reduced in PCa compared with NP by profiling of expressed sequence tags and quantitative RT-PCR analysis [28, 29]. However, there is no report, to our knowledge, in which *NBL1* expression in human systemic organs is compared and NBL1 expression in PCa and NP determined by immunohistochemical analysis has not been reported.

We compared *NBL1* mRNA expression between prostate and systemic normal organs. The average *NBL1* mRNA expression levels in NP and PCa were 19.9- and 8.1-fold greater, respectively, than in brain, in which *NBL1* is most highly expressed of the systemic normal organs. In comparison with that in the other systemic organs, the expression of *NBL1* mRNA is highly restricted to the prostate.

NBL1 Protein Expression in Cell Lysate and Culture Medium

To study whether NBL1 is a secreted protein, we performed Western blot analysis in 3 PCa cell lines. In cell lysate, moderate NBL1 expression was detected in DU145 cells as a band of approximately 27 kDa. LNCaP cells showed low NBL1 expression, and NBL1 expression was absent in PC3 cells (fig. 2a). In culture medium, very high NBL1 expression was noted in DU145 cells, low NBL1 ex-

Table 3. Summary of quantitative RT-PCR analysis of candidate genes specifically expressed in PCa and NP samples

Gene name	Name of normal organ with highest expression	PCa with highest mRNA expression level (A)	NP with highest mRNA expression level (B)	PCa specificity index (A/B)	PCa cases with mRNA expression level ≥10-fold normal organ with highest expression	NP cases with mRNA expression level ≥10-fold normal organ with highest expression
High specificity						
MSMB	Stomach	>100	>100	0.13	12	8
NBL1	Brain	29.6	71.2	0.42	5	5
AZGP1	Pancreas	21.5	22.4	0.96	1	2
Low specificity						
C1RL	Liver	7.7	8.2	0.94	0	0
NTN4	Pancreas	5.2	3.7	1.4	0	0
DMKN	Pancreas	2.1	7.0	0.3	0	0
No specificity						
FN1	Liver	0.78	0.37	2.1	0	0
COL4A5	Stomach	0.72	0.69	1.0	0	0
CCDC126	Skeletal muscle	0.58	0.07	8.3	0	0
SPP1	Kidney	0.56	0.34	1.6	0	0
CALU	Heart	0.32	0.26	1.2	0	0
CLU	Liver	0.3	1.0	0.3	0	0
SRGN	Bone marrow	0.27	0.67	0.4	0	0
FGB	Liver	<0.01	<0.01	–	0	0
TFPI	Kidney	<0.01	0.022	–	0	0
SFTPA1B	Lung	0.057	<0.01	–	0	0
ARMETL	Skeletal muscle	<0.01	<0.01	–	0	0

Target mRNA expression levels were standardized to 1.0 μg total RNA from the normal organ with the highest expression set as 1.0 (16 PCa and 9 NP samples).

pression was seen in LNCaP cells, and no expression of NBL1 was noted in PC3 cells. In the CAST analysis, colonies containing the *NBL1* gene were detected in the DU145 CAST library, indicating that Western blot analysis of NBL1 protein was consistent with CAST analysis. Next, we examined transition of NBL1 expression by Western blot analysis of cell extracts of DU145 transfected with *NBL1*-specific siRNAs. Two types of siRNAs (siRNA1 and siRNA2) and negative control siRNA were transfected into DU145 cell extracts. Expression of NBL1 in DU145 was suppressed by treatment with siRNA1 and siRNA2 in both cell lysate and culture medium (fig. 2b).

Immunohistochemical Analysis of NBL1 Expression in Normal Systemic Organs

We performed immunohistochemical analysis of NBL1 in 19 kinds of non-cancerous systemic tissues in 5 samples of each tissue. NBL1 expression was detected only in epithelium of the small intestine and colon, islets of the pancreas, and nerve cells in brain and spinal cord (fig. 3a–d). We did not detect the expression of NBL1 in heart, lung, esophagus, stomach, liver, spleen, pancreas, kidney, adrenal, ureter, bladder, testis, skin, skeletal muscle and vessel. When the level of NBL1 expression is compared in small intestine and colon, islets of the pancreas, and nerve cells in brain and spinal cord, expression in the brain nerve cells is higher than in the other tissues. These results are consistent with our quantitative RT-PCR results.

Immunohistochemical Analysis of NBL1 Expression in PCa and NP

We also performed immunohistochemical analysis of NBL1 in a total of 194 prostate samples, which included BPH in 13 patients and PCa in 181 patients. The clinical characteristics of the PCa patients are summarized in table 1. NBL1 staining was observed in the cytoplasm of both NP epithelium and cancer cells. In some acini, NBL1 expression was stronger at the apical side of luminal cells

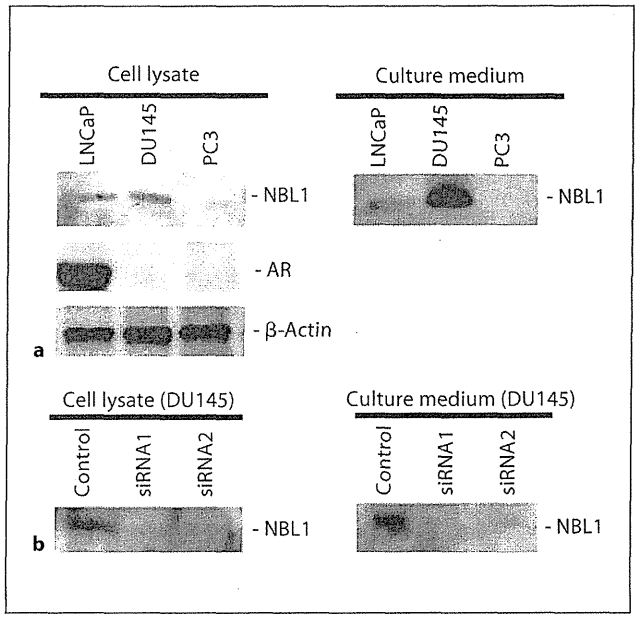


Fig. 2. Western blot analysis of NBL1 protein in PCa cell lines. NBL1 expression in DU145 and LNCaP cell lines was observed in cell lysate. In culture medium, high NBL1 expression was noted in DU145 cells (**a**). NBL1 expression in DU145 was suppressed by treatment with siRNA1 and siRNA2 in both cell lysate and culture medium (**b**).

and was detected in the prostatic ducts. Expression of NBL1 was not detected in stromal cells. Although NBL1 expression in PCa cells has some heterogeneity, NBL1 expression was detected in 1–95% of all samples (fig. 3e–h). All prostate samples were considered NBL1 positive if any cell stained positive. These immunohistochemical results suggest that NBL1 was highly expressed in and restricted to the prostate.

Next, we compared clinicopathological parameters with NBL1 expression scores in the prostate samples (fig. 4a–d). Mean NBL1 expression score was significantly higher in BPH samples and NP adjacent to PCa than in PCa itself ($p < 0.0001$). The mean NBL1 expression score was significantly higher in PCa classified as stage B than in PCa classified as stages C and D ($p = 0.0014$) and was significantly higher in PCa with Gleason score 6 than in PCa with Gleason score 7–10 ($p = 0.0024$). The mean NBL1 expression score was also significantly higher in PCa with PSA level ≤20 than in PCa with PSA level >20 ($p < 0.0001$).

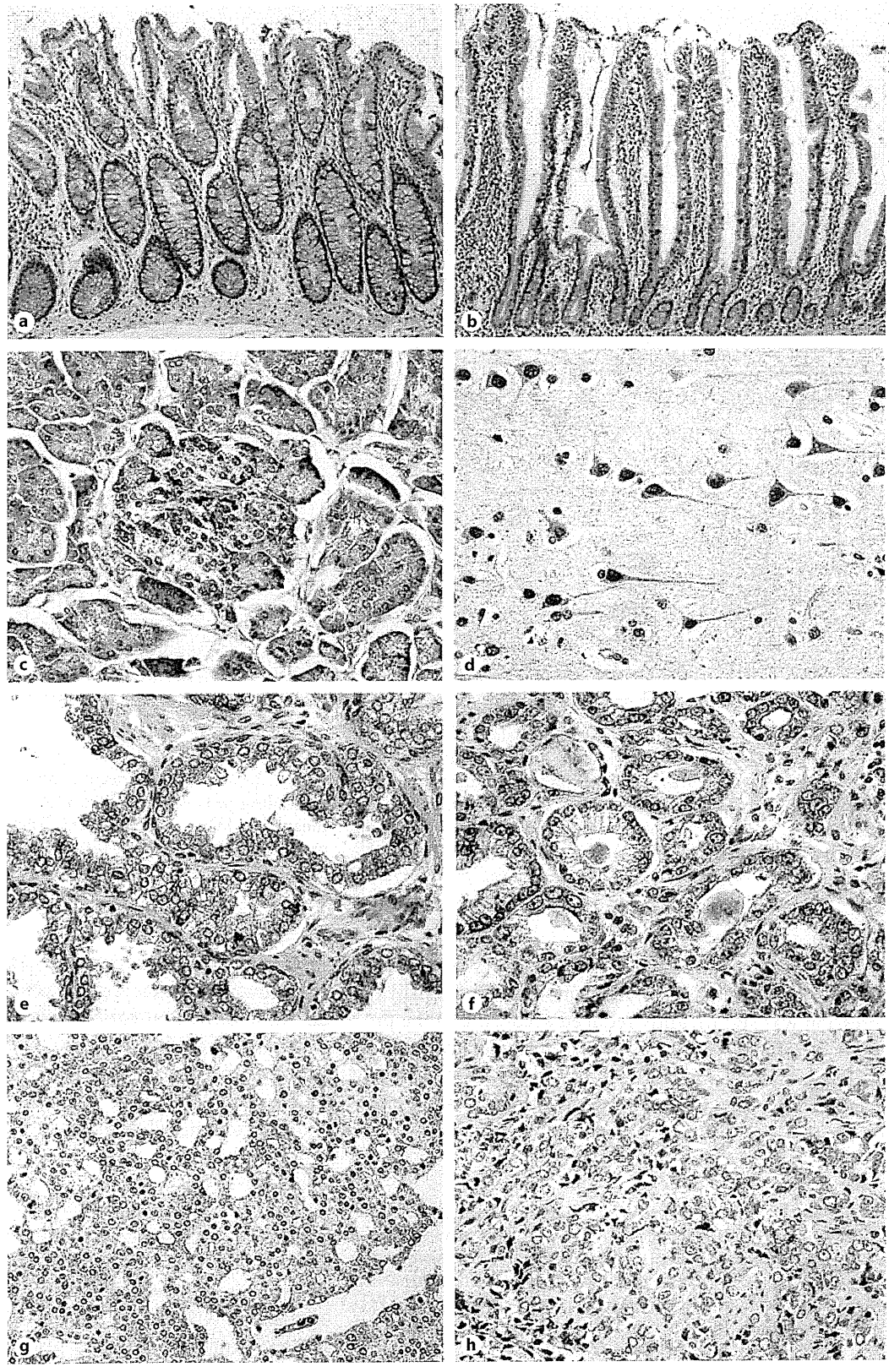
Effect of NBL1 Inhibition on Cell Growth and Invasive Activity of PCa Cells

We studied the biological role of NBL1 using the DU145 cell line because of the high expression of NBL1 in this cell line. To investigate the possible proliferative effects of NBL1 knockdown, we performed an MTT assay 4 days after siRNA transfection (fig. 4e). Cell viability was not significantly different between NBL1 siRNA-transfected DU145 and negative control siRNA-transfected DU145. Next, to determine the possible role of NBL1 in invasiveness, a transwell invasion assay was performed (fig. 4f). Invasion ability 1 day after siRNA transfection was not significantly different between NBL1 siRNA-transfected DU145 and negative control siRNA-transfected DU145.

Discussion

In the present study, we identified several genes that encode secreted proteins present in PCa and NP from CAST libraries. Quantitative RT-PCR revealed that *MSMB*, *NBL1* and *AZGP1* were expressed to a much higher extent in PCa and NP than in 14 types of normal tissues. MSMB, prostatic acid phosphatase and PSA are the three most abundant proteins found in semen. It was reported that MSMB also has high specificity for the prostate and has utility as a serum biomarker for PCa [24]. In addition, a single-nucleotide polymorphism of *MSMB* has been reported to increase the risk of developing PCa [25]. It was also reported that immunohistochemical staining of AZGP1 was a predictor of tumor recurrence and could be used as a specific serum biomarker for PCa [26, 27]. Although we could not detect PSA in our CAST library, we think that PSA might be one of the genes difficult to ligate into the pCAST vector. In contrast, little is known about NBL1 expression in PCa. NBL1 can stimulate differentiation of neuroblastoma cells in culture in the presence of retinoic acid [30], and its growth-suppressive activity has been noted in sarcoma cells [31]. NBL1 may play an important role in preventing cells from entering into the S phase [32]. *NBL1* mRNA was reported to be down-regulated in PCa compared with corresponding NP by quantitative RT-PCR analysis [28, 29].

Because the genes in the present study were identified by CAST analysis of PCa cell lines and quantitative RT-PCR analysis of bulk PCa and NP tissues, immunohistochemical analysis was required to determine which cells expressed these genes. With this analysis, we confirmed that NBL1 was highly expressed in the epithelium of PCa



Color version available online

3

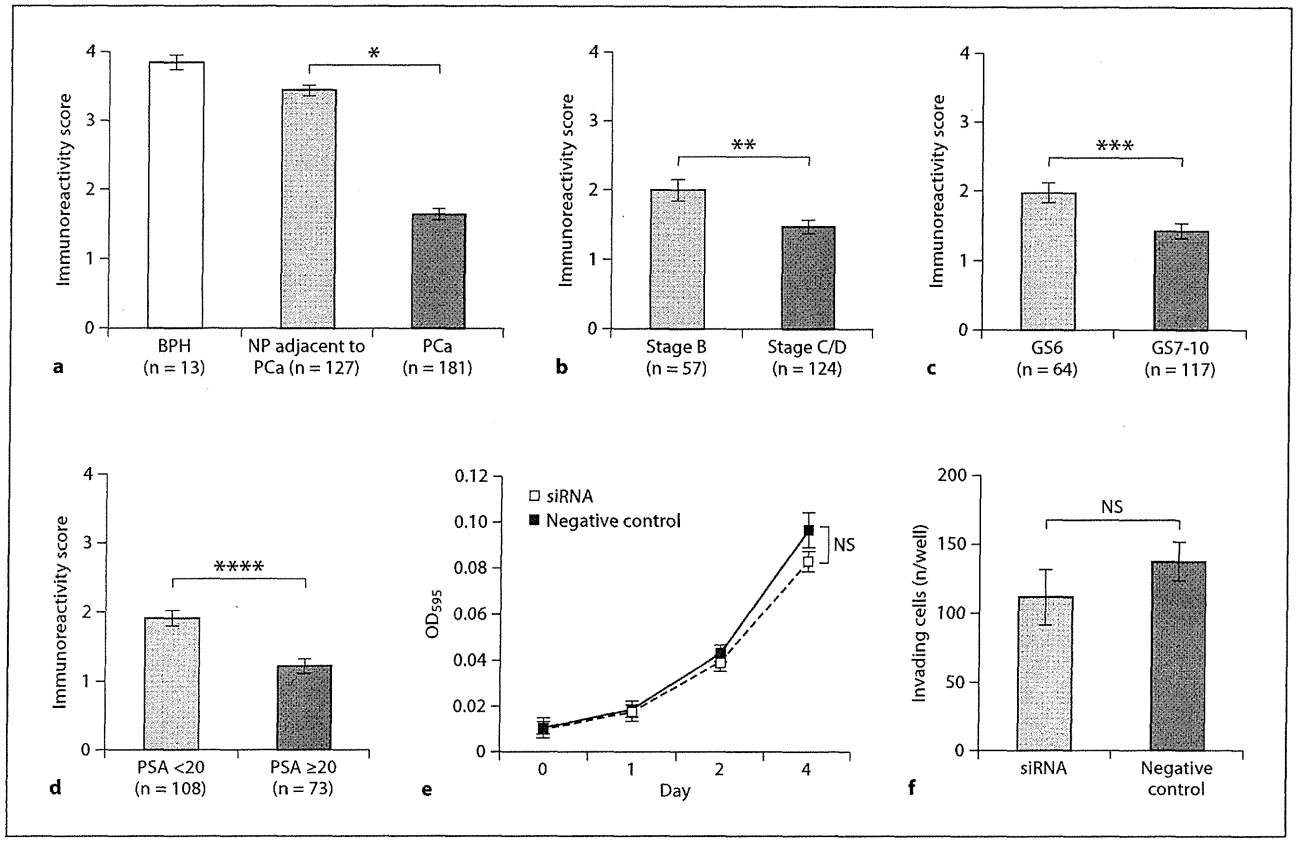


Fig. 4. NBL1 immunoreactivity scores in prostate. NBL1 expression score was higher in NP than in PCa. Means ± SEM. BPH (**a**). NBL1 expression score was higher in stage B than in stages C and D (**b**). NBL1 expression score was higher in PCa with Gleason score 6 than in PCa with Gleason score 7–10 (**c**). NBL1 expression score was higher in PCa with PSA ≤20 than in PCa with PSA >20 (**d**). **e**, **f** Effect of NBL1 knockdown on cell growth (**e**) and cell invasion (**f**) of DU145 cells. **e** Cell growth was assessed by an MTT assay 1, 2 and 4 days after seeding on 96-well plates in DU145 cells. **f** Invading cells were counted after 1 day. Bars and error bars, means and SD of three different experiments. NS = Not significant. * $p < 0.0001$; ** $p = 0.0014$; *** $p = 0.0024$; **** $p < 0.0001$.

and NP. In prostate samples, NBL1 expression was high in NP and lower in PCa. Furthermore, average NBL1 expression was significantly reduced according to the progression of stage, Gleason grade and preoperative PSA value. Because *NBL1* functions as a tumor suppressor gene, these results were consistent with those of previous reports. NBL1 expression was detected in epithelium of the intestine, pancreatic islets and nerve cells, but was absent in other non-cancerous systemic tissues and stromal cells in adult humans. Ozaki et al. [33] examined NBL1 expression in rat tissue by Northern blot analysis and showed that NBL1 was detected in brain, intestine, kidney and lung. They did not test NBL1 expression in rat prostate, but their results were similar to those of our present study in humans.

We also confirmed with Western blot analysis that high NBL1 expression was detected in DU145 cells in culture medium. Nakamura et al. [15] also reported that NBL1 was observed in the culture medium, and the amount of NBL1 secreted from the cells was calculated to be 80% of the total NBL1 protein. Furthermore, NBL1

Fig. 3. Immunohistochemical analysis of NBL1 in non-neoplastic human tissues and prostate tissues. NBL1 expression was detected in the epithelium of the small intestine and colon, islets of the pancreas and nerve cells, respectively (**a–d**). NBL1 staining was observed in the cytoplasm of normal prostate epithelium and PCa cells with Gleason scores 3, 4 and 5, respectively. NBL1 expression was also detected in prostatic ducts (**e–h**).

expression was detected in prostatic ducts in PCa and NP. Therefore, NBL1 may be secreted into semen as well as MSMB, prostatic acid phosphatase and PSA. PSA is produced by secretory epithelial cells in the acini and ducts, and it is secreted directly into the lumen. A characteristic feature of PCa is disruption of the basal cell layer and basement membrane, and this loss of the normal glandular architecture appears to allow PSA increased direct access to the peripheral circulation. PSA is normally found at lower levels in paraurethral and perianal glands, apocrine sweat glands, breast, thyroid and placenta, but these sites do not normally contribute measurable levels of PSA to the circulation [34]. Therefore, in spite of the fact that PSA expression is also higher in NP than in PCa in immunohistochemical analysis, serum PSA is increased in patients with PCa. Although establishment of an enzyme-linked immunosorbent assay system for serum samples is needed to clarify whether NBL1 can serve as a serum marker for detecting and monitoring PCa, we believe that NBL1 might be useful as a serum biomarker for PCa. NBL1 might be beneficial in addition to PSA in situations where PSA is less useful, such as in patients with low PSA level or castration-refractory disease.

The regulation of NBL1 is poorly understood, especially in PCa. Because *NBL1* is thought to be a tumor suppressor gene, it is thought that *NBL1* in PCa has mutations, deletions and methylation. Ozaki et al. [35] reported that two transcription sites were present in the rat NBL1 gene, suggesting the possibility of transcriptional regulation of NBL1. Further studies will clarify how NBL1 is regulated and whether androgen and the androgen receptor axis regulate NBL1. The biological function of the NBL1 protein is also poorly understood in PCa. NBL1 expression correlated with PCa progression, but NBL1 knockdown did not reduce viability and invasiveness relative to the negative control. Because NBL1 was also reported to act as a bone morphogenetic protein (BMP) antagonist by binding to BMPs [36] and BMPs are known to participate in the progression of PCa [37, 38], we speculate that interaction between NBL1 and BMP may play a more important role during growth and development in PCa than in cell cycle inhibition.

In summary, the present study yielded a list of genes that encode secreted proteins present in PCa and NP from CAST analysis. NBL1 expression is narrowly restricted to the prostate and is higher in NP than in PCa. Underexpression of NBL1 is associated with tumor progression. NBL1 has high potential as a biomarker of PCa and its progression.

Acknowledgments

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 provision 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; 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, Labor and Welfare of Japan, and in part by a grant (07-23911) from the Princess Takamatsu Cancer Research Fund.

References

1 Yasui W, Oue N, Kitadai Y, Nakayama H: Recent advances in molecular pathobiology of gastric carcinoma; in Kaminishi M, Takubo K, Mafune K (eds): The Diversity of Gastric Carcinoma Pathogenesis: Diagnosis, and Therapy. Tokyo, Springer, 2005, pp 51–71.
2 Yasui W, Oue N, Ito R, Kuraoka K, Nakayama H: Search for new biomarkers of gastric cancer through serial analysis of gene expression and its clinical implications. Cancer Sci 2004;95:385–392.
3 Hayashi T, Oue N, Sakamoto N, et al: 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. Pathobiology 2011;78:277–284.
4 Anami K, Oue N, Noguchi T, et al: Search for transmembrane protein in gastric cancer by the *Escherichia coli* ampicillin secretion trap: expression of DSC2 in gastric cancer with intestinal phenotype. J Pathol 2010;221:275–284.
5 Sirovich BE, Schwartz LM, Woloshin S: Screening men for prostate and colorectal cancer in the United States: does practice reflect the evidence? JAMA 2003;289:1414–1420.
6 Boyle P: Screening for prostate cancer: have you had your cholesterol measured? BJU Int 2003;92:191–199.
7 Carter HB: Prostate cancers in men with low PSA levels – must we find them? N Engl J Med 2004;350:2292–2294.
8 Gambert SR: Prostate cancer. When to offer screening in the primary care setting. Geriatrics 2001;56:22–26.
9 Edwards S, Campbell C, Flohr P, et al: Expression analysis onto microarrays of randomly selected cDNA clones highlights HOXB13 as a marker of human prostate cancer. Br J Cancer 2005;92:376–381.
10 Oefelein MG, Resnick MI: Effective testosterone suppression for patients with prostate cancer: is there a best castration? Urology 2003;62:207–213.
11 Chang SS, Kibel AS: The role of systemic cytotoxic therapy for prostate cancer. BJU Int 2009;103:8–17.

12 Ferguson DA, Muenster MR, Zang Q, et al: Selective identification of secreted and transmembrane breast cancer markers using *Escherichia coli* ampicillin secretion trap. Cancer Res 2005;65:8209–8217.
13 von Heijne G: A new method for predicting signal sequence cleavage sites. Nucleic Acids Res 1986;14:4683–4690.
14 Kadonaga JT, Gautier AE, Straus DR, Charles AD, Edge MD, Knowles JR: The role of the beta-lactamase signal sequence in the secretion of proteins by *Escherichia coli*. J Biol Chem 1984;259:2149–2154.
15 Nakamura Y, Ozaki T, Nakagawara A, Sakiyama S: A product of DAN, a novel candidate tumour suppressor gene, is secreted into culture medium and suppresses DNA synthesis. Eur J Cancer 1997;33:1986–1990.
16 Sobin LH, Wittekind CH (eds): TNM Classification of Malignant Tumors, ed 6. New York, Wiley-Liss, 2002, pp 184–187.
17 Eble JN, Sauter G, Epstein JI, et al: World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Urinary System and Male Genital Organs. Lyon, IARC Press, 2004.
18 Kondo T, Oue N, Yoshida K, et al: Expression of POT1 is associated with tumor stage and telomere length in gastric carcinoma. Cancer Res 2004;64:523–529.
19 Gibson UE, Heid CA, Williams PM: A novel method for real time quantitative RT-PCR. Genome Res 1996;6:995–1001.
20 Yasui W, Ayhan A, Kitadai Y, et al: Increased expression of p34cdc2 and its kinase activity in human gastric and colonic carcinomas. Int J Cancer 1993;53:36–41.
21 Ohtori S, Yamamoto T, Ino H, et al: Differential screening-selected gene aberrative in neuroblastoma protein modulates inflammatory pain in the spinal dorsal horn. Neuroscience 2002;110:579–586.
22 Sakamoto N, Oue N, Noguchi T, et al: Serial analysis of gene expression of esophageal squamous cell carcinoma: ADAMTS16 is upregulated in esophageal squamous cell carcinoma. Cancer Sci 2010;101:1038–1044.
23 Alley MC, Scudiero DA, Monks A, et al: Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res 1988;48:589–601.
24 Whitaker HC, Warren AY, Eeles R, Kote-Jarai Z, Neal DE: The potential value of microseminoprotein-β as a prostate cancer biomarker and therapeutic target. Prostate 2010;70:333–340.
25 Xu B, Wang J, Tong N, et al: A functional polymorphism in MSMB gene promoter is associated with prostate cancer risk and serum MSMB expression. Prostate 2010;70:1146–1152.
26 Henshall SM, Horvath LG, Quinn DI, et al: Zinc-α_2-glycoprotein expression as a predictor of metastatic prostate cancer following radical prostatectomy. J Natl Cancer Inst 2006;98:1420–1424.
27 Lapointe J, Li C, Higgins JP, et al: Gene expression profiling identifies clinically relevant subtypes of prostate cancer. Proc Natl Acad Sci USA 2004;101:811–816.
28 Asmann YW, Kosari F, Wang K, Cheville JC, Vasmatzis G: Identification of differentially expressed genes in normal and malignant prostate by electronic profiling of expressed sequence tags. Cancer Res 2002;62:3308–3314.
29 Shaikhibrahim Z, Lindstrot A, Buettner R, Wernert N: Analysis of laser-microdissected prostate cancer tissues reveals potential tumor markers. Int J Mol Med 2011;28:605–611.
30 Nakamura Y, Ozaki T, Ichimiya S, Nakagawara A, Sakiyama S: Ectopic expression of DAN enhances the retinoic acid-induced neuronal differentiation in human neuroblastoma cell lines. Biochem Biophys Res Commun 1998;243:722–726.
31 Hanaoka E, Ozaki T, Nakamura Y, Moriya H, Nakagawara A, Sakiyama S: Overexpression of DAN causes a growth suppression in p53-deficient SAOS-2 cells. Biochem Biophys Res Commun 2000;278:20–26.
32 Ozaki T, Nakayama Y, Enomoto H, Hirose M, Sakiyama S: Overexpression of DAN gene product in normal rat fibroblasts causes a retardation of the entry into the S phase. Cancer Res 1995;55:895–900.
33 Ozaki T, Sakiyama S: Molecular cloning and characterization of a cDNA showing negative regulation in v-src-transformed 3Y1 rat fibroblasts. Proc Natl Acad Sci USA 1993;90:2593–2597.
34 Balk SP, Ko YJ, Bubley GJ: Biology of prostate-specific antigen. J Clin Oncol 2003;21:383–391.
35 Ozaki T, Enomoto H, Nakamura Y, et al: The genomic analysis of human DAN gene. DNA Cell Biol 1997;16:1031–1039.
36 Kim AS, Pleasure SJ: Expression of the BMP antagonist Dan during murine forebrain development. Brain Res Dev Brain Res 2003;145:159–162.
37 Hamdy F, Autzen P, Robinson MC, Horne CH, Neal DE, Robson CN: Immunolocalization and messenger RNA expression of bone morphogenetic protein-6 in human benign and malignant prostate tissue. Cancer Res 1997;57:4427–4431.
38 Dai J, Keller J, Zhang J, Lu Y, Yao Z, Keller ET: Bone morphogenetic protein-6 promotes osteoblastic prostate cancer bone metastases through a dual mechanism. Cancer Res 2005;65:8274–8285.

Liver–intestine cadherin induction by epidermal growth factor receptor is associated with intestinal differentiation of gastric cancer

Naoya Sakamoto,[1] Naohide Oue,[1] Kazuhiro Sentani,[1] Katsuhiro Anami,[1] Naohiro Uraoka,[1] Yutaka Naito,[1] Htoo Zarni Oo,[1] Takao Hinoi,[2] Hideki Ohdan,[2] Kazuyoshi Yanagihara,[3] Kazuhiko Aoyagi,[4] Hiroki Sasaki[4] and Wataru Yasui[1,5]

Departments of [1]Molecular Pathology and [2]Surgery, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima; [3]Laboratory of Molecular Cell Biology, Department of Life Sciences, Yasuda Women's University Faculty of Pharmacy, Hiroshima; [4]Division of Genetics, National Cancer Research Institute, Tokyo, Japan

(Received January 27, 2012/Revised May 17, 2012/Accepted May 23, 2012/Accepted manuscript online June 7, 2012/Article first published online July 16, 2012)

Gastric cancer (GC) is one of the most common malignancies worldwide. The epidermal growth factor receptor (EGFR) molecule is very important in GC progression. To examine the correlation between EGFR and GC-related genes, we analyzed gene expression profiles of HT-29 cells treated with EGFR ligands and identified six genes upregulated by epidermal growth factor (EGF) and transforming growth factor (TGF)-α treatment. Among these, we focused on cadherin 17 (*CDH17*) encoding liver–intestine cadherin (LI-cadherin). Expression of LI-cadherin was induced by both EGF and TGF-α, as detected by quantitative RT-PCR and Western blot analysis. A luciferase assay showed that LI-cadherin promoter activity was enhanced by EGF or TGF-α in both HT-29 cells and MKN-74 GC cells. Immunohistochemical analysis of 152 GC cases showed that out of 58 LI-cadherin-positive cases, 24 (41%) cases were also positive for EGFR, whereas out of 94 LI-cadherin-negative cases, only 9 (10%) cases were positive for EGFR ($P < 0.0001$). Double-immunofluorescence staining revealed that EGFR and LI-cadherin were coexpressed. Significant correlation was found between LI-cadherin expression and advanced T grade and N grade. Both EGFR and LI-cadherin expression were more frequently found in GC cases with an intestinal mucin phenotype than in cases with a gastric mucin phenotype. These results indicate that, in addition to the known intestinal transcription factor caudal type homeobox 2, EGFR activation induces LI-cadherin expression and participates in intestinal differentiation of GC. (*Cancer Sci* 2012; 103: 1744–1750)

Gastric cancer remains a major public health issue as the fourth most common cancer and the second leading cause of cancer mortalities worldwide.[1] Gastric cancer is assumed to originate from a sequential accumulation of molecular and genetic alterations to stomach epithelial cells.[2] A molecular understanding of the genetics and epigenetics involved in GC pathogenesis may contribute to identifying novel GC biomarkers and highlight potential avenues for targeted therapies.

Epidermal growth factor and TGF-α both phosphorylate the EGFR and stimulate multiple signaling pathways involved in cell proliferation, anti-apoptosis, and other processes.[3–6] The overexpression of EGF and EGFR by various types of malignancies has been shown to correlate with metastasis, apoptosis, resistance to chemotherapy, and poor prognosis.[5,7,8] We previously reported that both EGF and EGFR are overexpressed in GC, and play a central role in tumor invasion and metastasis through an autocrine mechanism.[9–12] It is therefore important to gain a functional overview of EGFR signaling in GC.

In the present study, we used an oligonucleotide array analysis to generate a list of genes whose expression was induced by TGF-α or EGF treatment, and found that expression of *CDH17* was induced by EGFR ligands. *CDH17* was originally cloned from rat liver in 1994.[13] *CDH17* encodes LI-cadherin protein, which has similarity to the classic cadherins but is structurally distinct. Although the LI-cadherin name derives from the apparent expression pattern of the gene in the rat, in humans LI-cadherin is expressed almost exclusively in the small intestine and colon, but not in the liver.[13] Liver–intestine cadherin is one of the targets of CDX2, the caudal-related homeobox transcription factor. CDX2 has a key role in intestinal development and differentiation, therefore LI-cadherin may play a role in mediating CDX2 function in intestinal cell fate determination. Expression of TGF-α protein is detected in the top one-third of the intestinal crypt, which is composed only of terminally differentiated cells.[14] There are two major phenotypes of GC that are defined according to the mucin expression profile. We previously reported that LI-cadherin expression is associated with an intestinal mucin phenotype.[15] However, induction of gene expression related to intestinal differentiation of GC by EGFR activation has not been investigated. Here, we used luciferase assays to study whether EGFR activation affects *CDH17* transcription. Furthermore, EGFR and LI-cadherin expression were examined in surgically resected GC tissue by immunohistochemistry. The correlation between LI-cadherin expression and clinicopathological characteristics was analyzed.

Materials and Methods

Plasmids. Plasmids used in the present study were generated and described previously.[16] In brief, genomic DNA sequences corresponding to the promoter and 5′-flanking region of the human *CDH17* gene were cloned by PCR using genomic DNA purified from Caco2 cells. The *CDH17* PCR products were subcloned into the pGL4 basic vector (Promega, Madison, MD, USA). Polymerase chain reaction-based approaches were used to introduce mutations in the CDX2 binding sites in the pGL4 basic *CDH17* reporter gene construct. All inserts were verified by automated sequencing.

Cell lines and EGF/TGF-α treatment. The HT-29 colon cancer cell line was obtained from ATCC (Rockville, MD, USA) and maintained in DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% FBS in a humidified atmosphere of 5% CO_2

[5]To whom correspondence should be addressed.
E-mail: wyasui@hiroshima-u.ac.jp

doi: 10.1111/j.1349-7006.2012.02353.x

and 95% air at 37°C. MKN-74 was kindly provided by Dr Toshimitsu Suzuki (Niigata University School of Medicine, Niigata, Japan). HSC-39 and HSC-57 were established by Dr. Kazuyoshi Yanagihara. The cell line HT-29-CDX2 stably expresses CDX2, and HT-29-neo is the control cell line. These cell lines were maintained as described previously.[16] After 24 h of serum starvation, 1–100 nM concentrations of EGF (Sigma, St Louis, MO, USA) or TGF-α were added. The cells were treated for 48 h, and proteins and RNAs were then extracted.

Oligonucleotide array construction, hybridization, detection, and data analysis. The oligonucleotide array, Genopal (Mitsubishi Rayon, Tokyo, Japan), was prepared as described previously.[17] The array contained 208 genes, including GC-related genes identified by our previous SAGE analysis,[18] known genes related to the development and progression of GC,[19,20] genes related to DNA damage response and repair, and genes associated with sensitivity to anticancer drugs.[21] A list of the genes on the array is available upon request. Total RNA isolation, quantification and integrity of RNA assessment, hybridization, detection, and data analysis were carried out as described previously.[22]

Quantitative RT-PCR and Western blot analysis. Quantitative RT-PCR was carried out with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as described previously.[23] Sequence information of primers for CDX2 and CDH17 is available upon request. The *ACTB*-specific PCR products were amplified from the same RNA samples and served as an internal control. Primer sequences and additional PCR conditions are available upon request.

For Western blot analysis, cells were lysed as described previously.[24] The filter was incubated with primary anti-LI-cadherin antibody (goat polyclonal dilution 1:500 ; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Peroxidase-conjugated anti-goat IgG was used as the secondary probe. Immunocomplexes were visualized with an ECL Western Blot Detection System (Amersham Biosciences, Piscataway, NJ, USA). β-actin antibody (Sigma) was used as a loading control.

Luciferase assay. Forty-eight hours before transfection, cells were plated in 35-mm dishes. Transfection of cells at 30–50% confluency was carried out with 6 μL FuGENE6, 4 μg pGL4 containing LI-cadherin promoter constructs[16] and basic reporter gene construct, and 1 μg phRL-TK Renilla luciferase reporter vector (Promega). At 48 h after transfection, EGF and TGF-α treatment was carried out. At 48 h after treatment, cells were collected and resuspended in reporter lysis buffer (Promega). Luciferase activities were determined with luciferase assay reagent (Promega) and a GloMax luminometer (Promega).

Tissue samples. A total of 152 primary tumor samples were collected from patients diagnosed with GC. Patients were treated at the Hiroshima University Hospital (Hiroshima, Japan) or an affiliated hospital. Tumor staging was according to the TNM classification system. Because written informed consent was not obtained, for privacy protection, identifying information for all samples was removed before analysis. This was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government.

Immunohistochemistry. A Dako LSAB Kit (Dako, Carpinteria, CA, USA) was used for immunohistochemical analysis. In brief, microwave pretreatment in citrate buffer was carried out for 15 min to retrieve antigenicity. After peroxidase activity was blocked with 3% H_2O_2-methanol for 10 min, sections were incubated with normal goat serum (Dako) for 20 min to block non-specific antibody binding sites. Sections were incubated with mouse monoclonal anti-EGFR (1:20; Novocastra, Newcastle, UK) or goat polyclonal anti-LI-cadherin (1:50; Santa Cruz Biotechnology). After a 10-min incubation with substrate–chromogen solution, sections were counterstained with 0.1% hematoxylin. The percentage of stained cancer cells was evaluated for each antibody. A result was considered positive if at least 10% of the cells were stained. When fewer than 10% of cancer cells were stained, the immunostaining was considered negative.

For double-immunofluorescence staining, Alexa Fluor 546-conjugated anti-goat IgG and Alexa Fluor 488-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR, USA) were used as secondary antibodies.

Phenotypic analysis of GC. Gastric cancers were classified into four phenotypes: G type; I type; GI type; and N type. For phenotypic expression analysis of GC, we analyzed immunohistochemistry (as described above) with four antibodies, anti-MUC5AC, anti-MUC6, anti-MUC2, and anti-CD10 (all Novocastra). Gastric cancers in which more than 10% of cells in the section expressed at least one gastric epithelial cell marker (MUC5AC or MUC6) or intestinal epithelial cell marker (MUC2 or CD10) were classified as G type or I type cancers, respectively; sections that showed both gastric and intestinal phenotypes were classified as GI type; and those that lacked both gastric and intestinal phenotypes were classified as N type.

RNA interference and cell growth and *in vitro* invasion assays. To knock down endogenous *CDH17*, RNAi was carried out using *CDH17* and negative control siRNA oligonucleotides (Invitrogen). For MTT assays to monitor cell growth, cells were seeded at 2000 cells per well in 96-well plates.[25] Modified Boyden chamber assays were carried out to examine invasiveness. Transiently transfected cells were plated at 1×10^6 cells per well in RPMI-1640 medium with no serum in the upper chamber of a Transwell insert (8-μm pore diameter; Chemicon, Temecula, CA, USA) coated with Matrigel. Medium containing 10% serum was added in the bottom chamber. After incubation at 37°C for 24 and 48 h, 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 (Chemicon, Temecula, CA, USA) to assess the number of cells.

Statistical methods. Correlations between clinicopathologic parameters and LI-cadherin protein expression were analyzed by Fisher's exact test. $P < 0.05$ was considered statistically significant.

Results

Oligonucleotide microarray analysis. To identify GC-related genes whose expression was regulated by EGFR activation, we analyzed the gene expression profiles from TGF-α and EGF-treated HT-29 and non-treated HT-29. Previously, we showed activation of LI-cadherin expression by forced expression of CDX2 in the HT-29 colon cancer cell line;[16] however, in GC cell lines, forced expression of CDX2 could not activate LI-cadherin expression (Naohide Oue, unpublished data, 2010). Therefore, the correlation between EGFR and GC-related genes was investigated in HT-29 cells. Expression levels of 208 individual genes were compared between these two profiles. Six genes were identified that were expressed significantly higher (more than twice) in HT-29 cells treated with TGF-α or EGF, than in non-treated cells (Table 1). In EGFR ligand-treated HT-29 cells, no gene showed lower expression than in non-treated HT-29.

Epidermal growth factor receptor ligands induce LI-cadherin expression. Among six genes whose expression was upregulated in both TGF-α- and EGF-treated cells, we focused on *CDH17*. As shown in Figure 1(a), LI-cadherin expression detected by Western blotting was induced by EGF or TGF-α treatment in both HT-29 and MKN-74 cell lines. It has been

Table 1. Six genes upregulated by both epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) treatment in HT-29 cells

Symbol	Description	Genbank accession no.	Intensity		Fold change	Intensity		Fold change
			No treat	TGF-α		No treat	EGF	
VEGF	Vascular endothelial growth factor	NM_003376.3	3.9	9.1	2.4	3.9	17.3	4.5
CDH17	Cadherin 17, LI cadherin (liver–intestine)	NM_004063	2.7	6.2	2.3	2.7	7.7	2.9
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	NM_000389	15.2	39.8	2.6	15.2	41.8	2.7
IL8	Interleukin 8	NM_000584.2	7.7	26.3	3.4	7.7	16.2	2.1
CTSL	Cathepsin L	NM_001912	5.5	14.5	2.6	5.5	11.1	2.0
ABTB2	Ankyrin repeat and BTB (POT) domain containing 2	NM_145804.1	6.3	21.2	3.4	6.3	12.5	2.0

No treat, control cells that did not receive treatment.

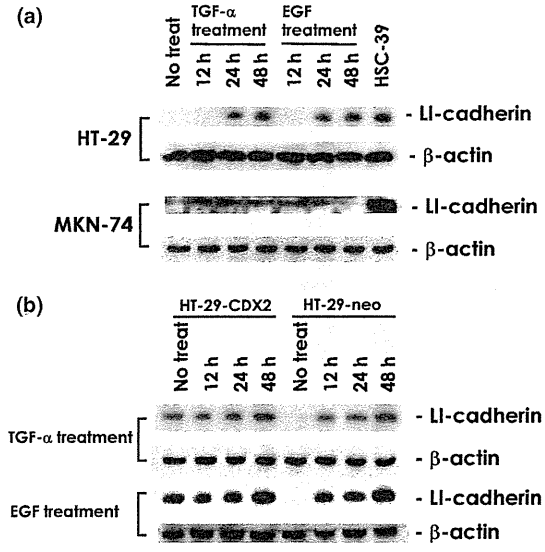


Fig. 1. Induction of liver–intestine cadherin (LI-cadherin) expression in cancer cells treated with epidermal growth factor receptor (EGFR) ligands. (a) LI-cadherin expression in HT-29 and MKN-74 cell lines after epidermal growth factor (EGF)/transforming growth factor-α (TGF-α treatment. (b) LI-cadherin expression in HT29-neo and HT29-caudal type homeobox 2 (CDX2) cell lines after EGF/TGF-α treatment. Western blot analysis confirmed temporal induction of LI-cadherin after EGF/TGF-α treatment, regardless of CDX2 expression.

reported that CDX2 regulates LI-cadherin expression, however, the potential interplay between CDX2 and EGFR signaling pathways has not been investigated. Expression of LI-cadherin in both HT-29-neo and HT-29-CDX2 cell lines was induced after TGF-α or EGF treatment; basal levels of LI-cadherin expression were higher in HT-29-CDX2 than in HT-29-neo cells (Fig. 1b). Quantitative RT-PCR analyses also revealed CDX2 expression was reduced and not induced after treatment with TGF-α or EGF. However, LI-cadherin expression was induced by treatment with TGF-α or EGF (Fig. 2a). A similar tendency was observed in HT-29-neo (data not shown) and HT-29-CDX2 (Fig. 2a) cells. We also examined MUC2, villin, and CD10 expression in HT-29 with TGF-α or EGF treatment. However, induction of these intestinal differentiation markers was not detected. These findings suggest that induction of LI-cadherin expression induced by TGF-α or EGF treatment may occur in a CDX2-independent manner. The induction of LI-cadherin in response to TGF-α or EGF treatment was then studied at a transcriptional level with an LI-cadherin promoter luciferase assay. HT-29 cells were cotransfected with a 0.5-kb human LI-cadherin promoter-driven luciferase construct and an SV40-directed Renilla construct as a control. At 48 h after transfection, cells were stimulated with TGF-α or EGF, resulting in a three and fourfold increase, respectively, in LI-cadherin promoter activity. In our previous observations, four CDX2 binding sites were found in the 0.5-kb human LI-cadherin promoter.[16] We also reported that when human LI-cadherin promoter-driven luciferase constructs with mutations in all four CDX2 binding sites were transfected, luciferase activity was eliminated. To investigate CDX2-independent induction of LI-cadherin by TGF-α or EGF treatment, HT-29 cells were transfected with a LI-cadherin promoter-driven luciferase construct with mutations in all four CDX2 binding sites. Following this transfection, cells stimulated with TGF-α or EGF also showed a three and fourfold increase, respectively, in LI-cadherin promoter activity (Fig. 2b). The similar upregulation of LI-cadherin promoter activity was observed in case of using HT-29-CDX2 (data not shown). We also examined LI-cadherin promoter activity using MKN-74 under the same conditions, and found that cells stimulated with TGF-α or EGF also showed a three and fourfold increase, respectively, in LI-cadherin promoter activity. This implies that EGFR signaling induced LI-cadherin transcription independently of CDX2.

Expression and distribution of EGFR and LI-cadherin in GC tissue. To examine the correlation between EGFR and LI-cadherin expression in GC tissue, we carried out immunohistochemical staining of LI-cadherin and EGFR in 152 surgically resected GC tissue samples. Staining of both LI-cadherin and EGFR occurred at the cell membrane. Of 152 GC cases, LI-cadherin and EGFR were expressed in 58 (38%) and 33 (22%) cases, respectively (Fig. 3a,b). In relation to CDX2, CDX2 expression was observed in almost all LI-cadherin-positive cases, and almost all gastric cancer cells simultaneously expressed CDX2 and LI-cadherin. However, LI-cadherin positive cell contained about a few CDX2 negative cells, CDX2-negative cells frequently expressed EGFR (Fig. 3a–c). In light of these findings, we suspect that EGFR contribute to induce LI-cadherin expression in GC at least partially. Coexpression of LI-cadherin and EGFR was observed in some GC cells by double-immunofluorescence staining (Fig. 3d). In EGFR-positive GC cells, expression of LI-cadherin was frequently found. However, in LI-cadherin-positive GC cells, EGFR was not always detected. In total, of 33 EGFR-positive cases, 24 (73%) cases were LI-cadherin-positive, whereas of 119 EGFR-negative cases, only 34 (29%) cases were LI-cadherin-positive ($P < 0.0001$, Table 2).

Clinicopathological characteristics of LI-cadherin-positive and EGFR-positive GC. The relationship of LI-cadherin staining to clinicopathological characteristics were investigated (Table 3). The LI-cadherin staining was observed more

doi: 10.1111/j.1349-7006.2012.02353.x

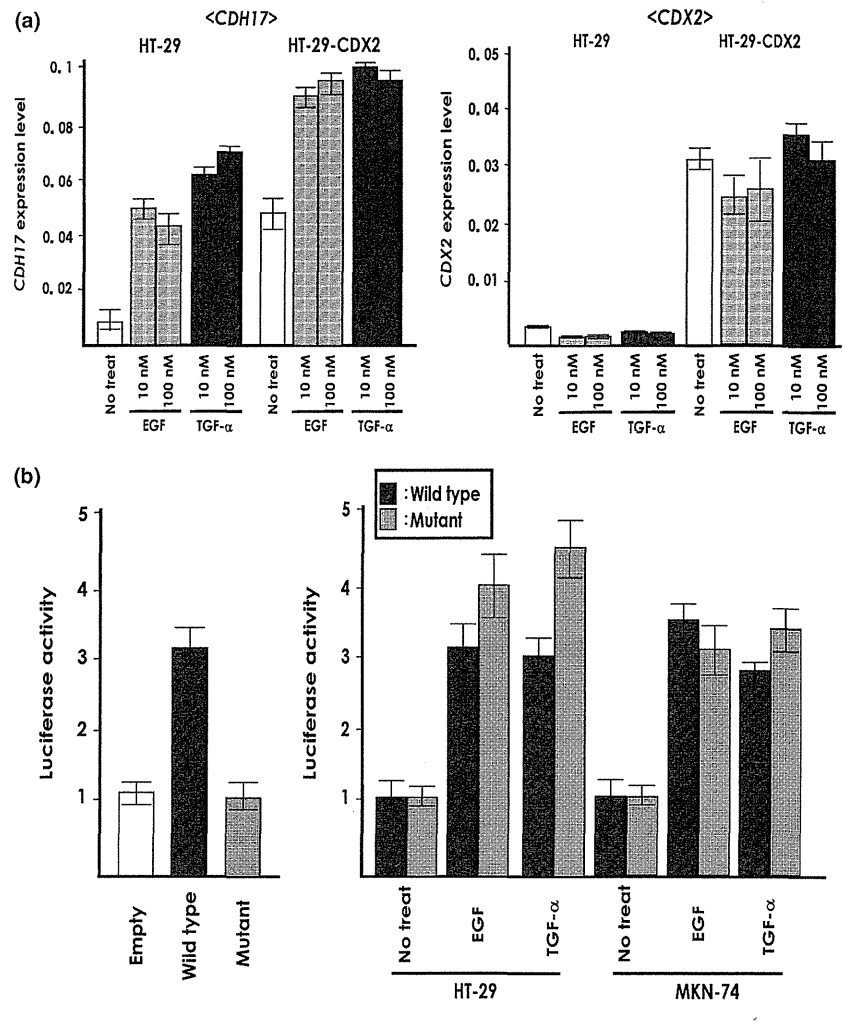


Fig. 2. Effect of epidermal growth factor (EGF)/transforming growth factor-α (TGF-α) treatment on cadherin 17 (*CDH17*) and caudal type homeobox 2 (*CDX2*) expression. (a) Quantitative RT-PCR analysis. Strong induction of *CDH17* was observed in both HT-29 and HT-29-CDX2 cells after EGF or TGF-α treatment without the upregulation of *CDX2*. (b) Liver–intestine cadherin (LI-cadherin) promoter reporter assays. Bars and error bars, mean and SE, respectively, of three different experiments. No treat, negative control. In the right-hand graph, luciferase activity of EGF and TGF-α were standardized by normalizing the strain that did not receive treatment (no treat) at 1.0. Empty, empty vector transfected HT-29 cells; Mutant, HT-29 or MKN-74 transfected with a LI-cadherin promoter-driven luciferase construct with mutations in all four CDX2 binding sites; Wild type, HT-29 or MKN-74 transfected with a LI-cadherin promoter-driven luciferase construct with no mutations in all CDX2 binding sites.

frequently in stage I/II cases (16/41, 39%) than in stage III/IV cases (6/39, 15%; $P = 0.0243$, Fisher's exact test). Moreover, LI-cadherin staining was detected more frequently in intestinal-type GC (20/46, 46%) than in diffuse-type GC (2/34, 6%; $P < 0.0001$, Fisher's exact test). In addition to the Lauren histology-based classification, GC can be subdivided into four phenotypes according to mucin expression. Gastric and intestinal markers were detected in 67 of 152 (44%) cases for MUC5AC, 16 (11%) cases for MUC6, 46 (31%) cases for MUC2, and 16 (11%) cases for CD10. We further investigated the association between LI-cadherin expression and mucin phenotype, because LI-cadherin was detected in intestinal metaplasia of the stomach and colon. Expression of both EGFR and LI-cadherin was found more frequently in GC of I and GI types than GC of G and GI types (Fig. 3d). In the group of 59 advanced GC patients, EGFR expression had significant prognostic impact. However, no significant prognostic impact was found for LI-cadherin expression in GC cases (data not shown).

Effect of LI-cadherin inhibition on cell growth and invasive activity. The LI-cadherin-positive GC cases were observed more frequently in stage III/IV than in stage I/II, suggesting that LI-cadherin could be associated with tumor progression. However, the biological significance of LI-cadherin in GC has not been studied. We carried out an MTT assay 8 days after LI-cadherin siRNA transfection in the HSC-57 cell line. HSC-57 cells were selected for high LI-cadherin expression. We confirmed that LI-cadherin siRNA-transfected HSC-57 cells showed significantly reduced LI-cadherin expression (data not shown). Cell viability was not significantly different between LI-cadherin siRNA-transfected and negative control GC cells (data not shown). To determine the possible role of LI-cadherin in GC cell invasiveness, a Transwell invasion assay was carried out in the HSC-57 cell line. There was no significant difference in invasion between LI-cadherin knockdown and negative control GC cells (data not shown).

Discussion

In the present study, by using a customized oligonucleotide microarray, we identified six GC-related genes (*VEGF*, *CDH17*, *CDKN1*, *IL8*, *CTSL*, and *ABTB2*) whose expression was upregulated by EGFR activation. Among these genes, we successfully showed that EGFR activation induced LI-cadherin expression. In addition, LI-cadherin induction occurred in a CDX2-independent manner, and additional induction of LI-cadherin expression was observed in CDX2-transfected cells. These results suggest that high expression of LI-cadherin is required for EGFR activation as well as CDX2 expression. Because the EGFR signaling pathway forms a wide-ranging network, it remains unclear which component of the signaling pathway directly regulates LI-cadherin. This requires further study.

In normal colon, expression of EGFR and LI-cadherin was found. Both TGF-α and $p21^{waf1/cip1}$ protein expression is only detected in the top one-third of the crypt, which is only composed of terminally differentiated cells.[26,27] In the present study, expression of $p21^{waf1/cip1}$ was also induced by TGF-α

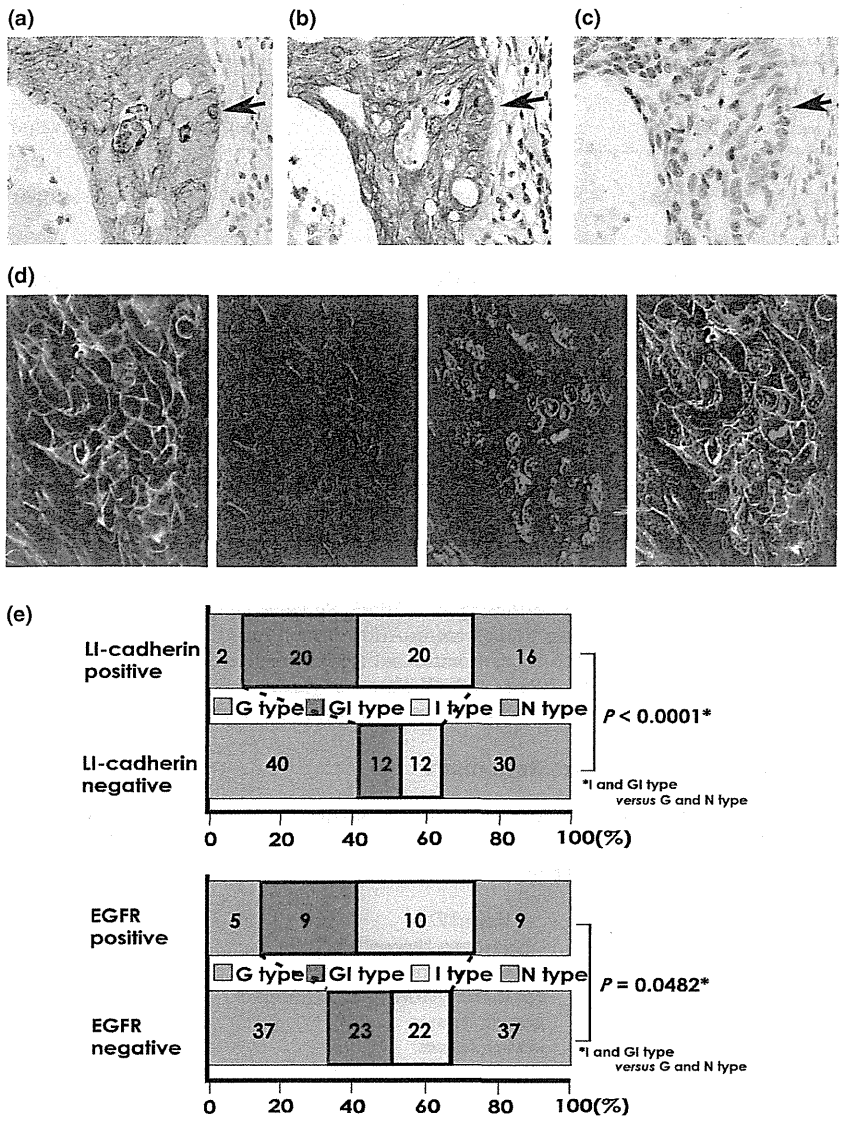


Fig. 3. Immunohistochemical analysis of epidermal growth factor receptor (EGFR) and liver–intestine cadherin (LI-cadherin) in gastric cancer (GC) and phenotypic analysis of EGFR- and LI-cadherin-positive GC cases. Expression pattern of EGFR (a), caudal type homeobox 2 (CDX2) (b), and LI-cadherin (c) (original magnification, ×400). Arrows indicate EGFR-positive (a), LI-cadherin-positive (b), and CDX2-negative (c) GC cells. (d) Double immunofluorescence staining shows that EGFR and LI-cadherin were coexpressed in some GC cells. Blue, DAPI; green, EGFR; red, LI-cadherin. (e) Distribution of gastric (G), intestinal (I), gastric and intestinal mixed (GI), and unclassified (N) phenotypes of GC in EGFR- and LI-cadherin-positive cases. Both EGFR and LI-cadherin expression were more frequently found in GC with intestinal features (I or GI type) than the others. *P*-values were analyzed by Fisher's exact test.

Table 2. Expression of liver–intestine cadherin (LI-cadherin) and epidermal growth factor receptor (EGFR) in gastric cancer tissue

	EGFR		*P*-value†
	Positive	Negative	
LI-cadherin			
Positive	24	34	<0.0001
Negative	9	85	

†Fisher's exact test.

and EGF treatment (data not shown). It has been reported that $p21^{waf1/cip1}$ is associated with the processes of cell-cycle arrest, apoptosis, and differentiation.[28] Taken together, these results suggest that EGFR activation induces intestinal differentiation. High expression of LI-cadherin may have some effect on terminal differentiation of colonic epithelial cells. However, despite induction of $p21^{waf1/cip1}$ expression, cell growth activity was upregulated in HT-29 cells (data not shown). To clarify whether $p21^{waf1/cip1}$ is involved in cell growth inhibition of HT-29, further studies are required.

In GC, LI-cadherin overexpression has been reported to be associated with lymph node metastasis.[29] In contrast, in colorectal cancer, reduced expression of LI-cadherin is frequently found in cases with lymph node metastasis or poor survival. Thus, the functions of LI-cadherin in human cancers are controversial and unclear. In the present study, we carried out MTT and Transwell invasion assays after LI-cadherin knockdown in the HSC-57 cell line. However, cell viability and invasion ability were not significantly altered. To clarify the biological and clinical significance of EGFR and LI-cadherin expression in GC, we examined the expression of these two molecules in GC tissue through immunohistochemistry and compared this with clinicopathologic parameters, prognosis, and mucin phenotype. Our data for the relation between molecular expression and tumor stage in all of our GC cases were consistent with the results of previous studies.[30] With regard to mucin phenotype, both EGFR and LI-cadherin expression were detected more frequently in I-type GC, and this finding is in accordance with previous studies.[15] Many studies have implicated the EGFR signaling pathway in the regulation of intestinal epithelial cell growth and differentiation.[31–33] Animals that are EGFR-null die early in postnatal life and show severe defects in intestinal cell proliferation and organization, along with many other abnormalities.[31] Overexpression or mutation of EGFR has been associated with many different carcinomas, including colonic carcinoma.[34] These

doi: 10.1111/j.1349-7006.2012.02353.x