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Molecular pathology of gastric cancer: Research and practice

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ABSTRACT

Recent advances in the understanding of molecular stomach carcinogenesis are reviewed. As to molecular events in individual mucin phenotypes of gastric cancer, the CDX2-Reg IV-SOX9 pathway is associated with the intestinal mucin phenotype, while OLFM4 and CLDN18 are novel markers for the gastric phenotype. microRNAs play an important role in epigenetic deregulation in gastric cancer. Many microRNAs are up-regulated and down-regulated, and some of these are associated with histological differentiation and cancer progression. Reduced miR-200 may participate in the genesis of diffuse type gastric cancer by reducing E-cadherin expression. Genetic polymorphism is a crucial endogenous cause and a fundamental factor of cancer risk. PSCA polymorphism alters the susceptibility to diffuse type gastric cancer through modulation of cell proliferation activity. Cancer stem cells possess the capacity for self-renewal and cause the heterogeneous lineages of cancer cells. Cancer stem cells also show resistance to antitumor chemotherapy. Only a minor population of gastric cancer cells reveals the properties of cancer stem cells, and CD44 is one of the markers for gastric cancer stem cells. The origin of gastric cancer stem cells remains to be elucidated.

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Introduction

Gastric cancer is one of the most common cancers worldwide, and mortality due to gastric cancer is second next to lung cancer. Cancer develops as a result of an accumulation of various endogenous and exogenous causes. Dietary factors and *Helicobacter pylori* (*H. pylori*) infection are important exogenous causes for gastric cancer, while many genetic polymorphisms are found to be associated with predisposition to cancer development. Multiple genetic and epigenetic alterations occur in the course of carcinogenesis and progression of gastric cancer [56,58,59,61,62]. These include telomerase activation, genetic instability, and abnormalities in oncogenes, tumor suppressor genes, growth factors, matrix degradation enzymes, cell cycle regulators, cell adhesion molecules, etc. Gastric cancers are histologically classified into "differentiated" and "undifferentiated" types, or "intestinal" and "diffuse" types based on the glandular structure, while these can also be classified into "intestinal" and "gastric" types on the basis of the mucin expression profile [48]. Some of the genetic and epigenetic changes differ depending on the histological type or mucin phenotype. Advances in genomic science have made it possible to uncover detailed mechanisms of molecular stomach carcinogenesis. In recent years, microRNA (miRNA) was believed to play a crucial role in the cellular process by regulating gene expression and to participate deeply in epigenetic alterations in gastric cancer [8]. Furthermore, a recent focus of attention was the presence of "cancer stem cells" among heterogeneous cancer cells and its therapeutic implication [21].

This review describes the molecular carcinogenesis of "intestinal" and "gastric" types of gastric cancer, the importance of miRNAs, the significance of genetic polymorphism, and the role of cancer stem cells in gastric cancer.

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Molecular carcinogenesis of intestinal and gastric type gastric cancer

In addition to the histological classification, such as "differentiated" and "undifferentiated" types, or "intestinal" and "diffuse" types based on glandular structure, gastric cancers are also classified into four types: G (gastric) type, I (intestinal) type, GI (gastric and intestinal mixed) type, and N (neither gastric nor intestinal) type, on the basis of the mucin expression, including MUC5AC, MUC6, MUC2 and CD10 [48]. It has been shown that *sry*-related high-mobility group (HMG) box 2 (SOX2) is stomach-specific, and caudal-type homeobox (CDX) 1 and CDX2 are intestine-specific transcription factors [2,50]. This classification is important, because some molecular events cause differences between the G type and I type; DNA methylation of the mismatch repair gene and microsatellite instability are frequent in G type, and p53 mutation occurs frequently in I type [24,41]. It has also been suggested that in comparison with I type, G type is biologically aggressive [45].

We have performed transcriptome dissection of gastric cancer through serial analysis of gene expression (SAGE) and found several novel genes associated with G type and I type gastric cancers [34,57]. SAGE allows for the global analysis of gene expression in a quantitative manner, without prior knowledge of the exact sequence of the genes. Our SAGE library is one of the largest gastric cancer libraries in the world, and the sequence data are publicly available at SAGEmap (GEO accession number GSE 545, SAGE Hiroshima gastric cancer tissue) [34]. By comparing SAGE data between gastric cancers and normal gastric tissue in combination with quantitative RT-PCR and immunohistochemistry, several genes, such as Reg IV, OLFM4, and CLDN18, were found to show mucin phenotype-specific expression [61].

Reg IV (regenerating islet-derived family, member 4) belongs to the calcium-dependent lectin superfamily, and was originally isolated as a gene up-regulated in inflammatory bowel diseases [18]. Reg IV is expressed in about 30% of gastric cancers, and Reg IV protein is detectable in sera of about 30% of gastric cancer patients [31]. Forced expression of Reg IV in gastric cancer cell lines induced the expression of the phosphorylated form of the EGF receptor, Bcl-2, Bcl-XL, survivin, and the phosphorylated form of AKT. Mucin-like staining of Reg IV in gastric cancer was significantly associated with MUC2-positive I type. CDX2 induced the expression of Reg IV. Therefore, CDX2 is an up-stream regulator for Reg IV expression [61]. SOX9 was found to be one of the down-stream targets of Reg IV. SOX9 is a member of the SOX [*sry*-related high-mobility group box] family of HMG DNA-binding domain transcription factors, and is required for the development and differentiation of multiple cell lineages [40]. Many cancer cells of I type are positive for SOX9, and SOX9-positive tumor cells co-localize with Reg IV-positive cells. CDH17 is also associated with I type gastric cancer. CDH17 encodes cadherin-17, also referred to as liver–intestine (LI)-cadherin, and is a structurally unique member of the cadherin superfamily [11]. A significant association was observed between CDH17 and CDX2 expression, as well as MUC2-positive I type gastric cancer. CDH17 is induced by CDX2 and also by EGFR activation in gastric cancer cells. HOXA10 is a member of the homeobox gene superfamily of transcription factors that contain a helix-turn-helix DNA-binding motif, and participates in myeloid cell differentiation and proliferation [25]. HOXA10 is also associated with MUC2-positive I type gastric cancers. The role of HOXA10 in stomach carcinogenesis remains unknown,

Olfactomedin 4 (OLFM4), also referred to as GW112 or human G-CSF clone-1 (hGC-1), was originally cloned from human myeloid cells and may facilitate apoptosis, tumor growth, and invasion [28,63,64]. In the non-neoplastic gastrointestinal tract, OLFM4 is expressed in tall columnar epithelial cells at the bottom of the intestinal crypt where the Lrg-5 (leucine-rich-repeat containing G-protein-coupled receptor 5)-positive stem cells are located [35,53]. Strong OLFM4 expression was detected in 60% of gastric cancers, and a significant association was found with the differentiated type of gastric cancer and Reg IV-negative tumors [35]. OLFM4 expression was more frequently observed in G type than in other types (I, GI and null). We have confirmed that combined measurement of Reg IV and OLFM4 protein levels in sera revealed a sensitivity of 57% for the detection of gastric cancer [35]. During the course of the search for novel tumor suppressor genes by SAGE data analysis, CLDN18 (encoding claudin-18) was identified to be preserved in G type gastric cancer [38]. Claudins comprising 24 members (claudins 1–24) are components of tight junction strands and are expressed in an organ-specific manner [51]. CLDN18 was first identified as a down-stream target of the T/EBP/NKX2.1 homeodomain transcription factor and has two variants: lung-specific variant 1 and stomach-specific variant 2 [32]. CLDN18 is expressed in the cell membrane of all epithelial cells of the normal gastric mucosa. The expression of CLDN18 is lost in MUC2-positive I type, while MUC5AC-positive G type preserves CLDN18 expression. It is necessary for a functional analysis to be performed; preservation of CLDN18 may participate in the genesis of G type gastric cancer.

Epigenetic regulation by microRNAs and its pathobiological role in gastric cancer

Various epigenetic alterations are involved in stomach carcinogenesis. Of these, DNA methylation associated with histone modification and chromatin remodeling to inactivate tumor suppressor genes has been intensively studied [60]. A recent focus of attention was the role of microRNA in cancer development and progression [8]. miRNA is a new class of small non-coding RNAs of 19–25 nucleotides which are cleaved from 60 to 110-nucleotide pre-miRNA precursors by RNase III Dicer [5]. Single-stranded miRNAs bind through partial sequence homology to the 3′ untranslated region of potentially hundreds of target genes and cause degradation of mRNAs and inhibition of translation. Over 30% of human genes are believed to be regulated by this mechanism. While initial studies suggested that miRNAs generally function as tumor suppressors, recent evidence has revealed that miRNAs possess either anti-tumorigenic or oncogenic properties depending on target genes [42]. In the last two years, more than 40 papers on miRNA in gastric cancer have been published. These include down-regulation of miR-9, miR-31, miR-141, miR-143, miR-145, and miR-433, as well as up-regulation of miR-34, miR-128, and miR-421 in gastric cancer.

Ueda et al. [52], in collaboration with us, studied the relationship between miRNA expression and progression and prognosis of gastric cancer using 182 Japanese gastric cancer samples. Using miRNA microarray analysis, 22 miRNAs were up-regulated and 13 were down-regulated in gastric cancer in comparison with corresponding non-neoplastic gastric tissue. Up-regulated miRNAs include miR-181d, miR-181a, miR-181c, miR-181b, miR-21, miR-25, miR-92, miR-93, miR-17-5p, miR-106a, miR-20b, miR-135a, miR-425, miR-106b, miR-20a, miR-19b, miR-224, miR-18a, miR-135b, miR-19a, miR-345, and miR-191, while down-regulated miRNAs include miR-148a, miR-148b, miR-375, miR-29b, miR-29c, miR-152, miR-218, miR-451, miR-30d, miR-30a, miR-30b, miR-30c, and miR-422b. In relation to the histological classification of gastric cancer, miR-105, miR-100, miR-125b, miR-199a, miR-99a, miR-143, miR-145, and miR-133a are up-regulated in the diffuse type, whereas miR-373, miR-498, miR-202, and miR-494 are up-regulated in the intestinal type. miR-125b, miR-199a, and miR-433 are the most important miRNAs involved in cancer progression. Low expression of let-7 g and miR-433 and high expression of miR-214 are independent unfavorable prognostic markers in

multivariable analysis. We independently examined the expression profile of miRNAs by microarray and found that miR-200a, miR-200b, miR-200c, and miR-141 were down-regulated in diffuse type gastric cancer. It is recognized that epithelial–mesenchymal transition (EMT) is an important process to form diffuse histology and initiate metastasis by enhancing the motility of tumor cells. In transforming growth factor-beta (TGF-beta)-induced EMT in murine mammary epithelial cell system, members of the miR-200 family are repressed during EMT. Overexpression of miR-200 hinders EMT by enhancing E-cadherin expression through direct targeting of ZEB1 and ZEB2, which encode transcriptional repressors of E-cadherin [7,22]. Therefore, reduced expression of miR-200 family may participate in the genesis of diffuse histology of gastric cancer by reducing E-cadherin expression through ZEB1 and ZEB2. On the other hand, we have previously demonstrated that 40% of gastric cancers overexpress E2F-1, a master transcription factor for cell cycle control [44]. It has been recently reported in gastric cancer that E2F-1 up-regulates miR-106b, miR-93, and miR-25, a cluster of intronic miRNAs hosted in Mcm7 (Minichromosome maintenance 7) gene, while miR-106b and miR-93 control E2F-1 expression, establishing a negative feedback loop [37]. Furthermore, these miRNAs impair TGF-beta-dependent cell cycle arrest and apoptosis by inhibiting the synthesis of $p21^{WAF1/CIP1}$ and Bim (Bcl-2-interacting mediator of cell death). Therefore, this is one of the mechanisms for TGF-beta resistance frequently found in gastric cancer besides the abnormalities in TGF-beta receptor-signaling pathway.

It is known that miRNA genes are also transcriptionally regulated by DNA methylation and chromatin remodeling as in protein-coding genes [39]. In stomach carcinogenesis, a mucosal field with *H. pylori* infection is believed to be a condition predisposed to cancer development. Ando et al. [3] studied methylation levels of three miRNA genes (miR-124a-1, miR-124a-2, and miR-124a-3) in gastric mucosa with and without *H. pylori* infection from individuals with and without gastric cancer and in gastric cancer tissues. In gastric mucosa taken from healthy individuals, the methylation levels were significantly higher with *H. pylori* infection than without, while in gastric mucosa without *H. pylori* infection, the methylation levels were significantly higher in non-cancerous gastric mucosa taken from gastric cancer patients than in those from healthy individuals. The methylation levels in gastric cancers were highly variable. From these findings, they suggest that methylation-silencing of miRNA genes, in addition to that of protein-coding genes, contribute to the formation of field defect (predisposed mucosal field) to gastric cancer.

Genetic polymorphism and gastric cancer risk

Genetic factors, in addition to environmental factors, substantially contribute to the development and progression of gastric cancer. Gonzalez et al. [14] described that genetic susceptibility must be crucial in a variety of processes relevant to gastric carcinogenesis, including (1) the mucosal protection in the face of *H. pylori* infection and other carcinogens; (2) the inflammatory response, which conditions the maintenance, severity, and outcome of the *H. pylori* infection; (3) the functioning of carcinogen detoxification and antioxidant protection; (4) the intrinsic variability of DNA repair processes; and (5) cell proliferation ability. For instance, variants of IL-1beta (*IL1B*) and IL-1 receptor antagonist (*IL1RN*) genes, *IL1B* (-31 T genotype), and *IL1RN* IVS 86 bp VNTR (2/2 genotype) increase IL-1beta production and inhibit gastric acid secretion [12]. These are associated with an increased risk of chronic hypochlorhydric response to *H. pylori* infection and an increased gastric cancer risk. Hamajima et al. [16] reviewed genetic factors involved in the development of *H. pylori*-related gastric cancer. Genetic polymorphisms of tumor necrosis factor-alpha gene (TNF-A) and possibly quinone oxidoreductase 1 (NQO1) are also associated with *H. pylori* infection. Upon *H. pylori* infection in gastric epithelial cells, injected CagA is phosphorylated by src family kinases, interacts with src homology 2 domain-containing protein tyrosine phosphatase (SHP-2), and transduces signal to downstream molecules participating in atrophic gastritis [19]. Frequent G/A SNP in the intron 3 of the PTPN11 gene encoding SHP2 is associated with gastric atrophy monitored by serum pepsinogen I/II in the Asian population but not in Caucasians [15].

As to cancer-related genes and cancer risk, we have reported that single nucleotide polymorphism (SNP) in the transmembrane domain of the *HER-2/c-erb*B2 (655 Ile > Val, A > G) significantly affects gastric cancer risk [23]. SNP in the promoter regions of the *MMP-9* (-1562C/T) does not alter cancer susceptibility but is associated with tumor invasion, metastasis, or stage grouping [29]. Other polymorphisms of cancer-related genes reported to show a significant association with gastric cancer risk include cyclin D1, CDH1 (E-cadherin), EGFR, $p16^{INK4A}$, $p21^{WAF1/CIP1}$, etc. [12]. Recently, the Study Group of Millennium Genome Project for Cancer, including us [49], reported that genetic variation in prostate stem cell antigen (PSCA) is associated with susceptibility to diffuse type gastric cancer. A two-stage genome-wide association study in Japan has identified a significant association between an intronic SNP (rs2976392) in PSCA and diffuse type gastric cancer, but the association is far less significant with the intestinal type. PSCA is expressed in differentiating gastric epithelial cells and is frequently silenced in gastric cancer. PSCA inhibits cell proliferation *in vitro*. Substitution of C with the risk allele T at a SNP (rs2294008) in the first exon reduces transcriptional activity. The same risk allele was also associated with diffuse type gastric cancer in Korea.

In addition to affecting cancer risk, genetic polymorphisms are also associated with therapeutic efficacy and toxicity of anti-cancer drugs [55]. The genotype of CYP2C19 influences the eradication rate of *H. pylori*, and is useful to predict the success of the treatment [43]. An important implication regarding chemotherapy is that the genotype of UDP-glucuronosyltransferase 1A1 (UGT1A1) gene affects the severity of toxicity during irinotecan therapy [20].

Cancer stem cells in gastric cancer

Although initiation of cancer cell may occur at single cell levels, most of cancers consist of heterogeneous cancer cell populations mimicking the hierarchy of stem cell lineage. The cancer stem cell hypothesis is not entirely new, but only recently, actual features of cancer stem cells have been characterized by advanced technology [21]. The existence of cancer stem cells was first proven in acute myeloid leukemia in 1997 [6] and verified in a variety of solid tumors, such as cancers of the breast, brain, prostate, pancreas, etc. [21]. As few as 100 cancer stem cells can grow and form tumor in immunodeficient mice [46]. Cancer stem cells are now defined as "cells within a tumor that possess the capacity for self-renewal and that can cause the heterogeneous lineages of cancer cells that constitute the tumor" [9]. Another important aspect of cancer stem cells is the resistance to anti-tumor therapy. A variety of cancer stem cells express ATP-binding cassette family of transporter proteins to pump out multiple chemotherapeutic drugs.

As to gastric cancer, Haraguchi et al. [17] initially studied cancer stem cells by using a flow cytometry-based side population (SP) technique with Hoechst 33342 dye, and found that SP cells occupied 0.6–2.2% of gastric cancer cells. Because SP is highly enriched in stem cells, this is a useful tool for stem cell studies if specific cell surface markers are unknown. The SP cells express ATP-binding cassette, subfamily G, group 2 (ABCG2) and ATP-binding cassette, subfamily B, member 1 (ABCB1, also referred to as MDR-1 or P-glycoprotein) and show evidence of chemoresistance, self-renewal

with the ability to differentiate progeny, and high tumorigenicity [13,17]. The SP cells of gastric cancer express a variety of adhesion molecules at high levels and possess a high potential for peritoneal metastasis [33].

Identification of a specific cell surface marker for cancer stem cells is important to study its biology and clinical implication. Many cell surface markers have been identified in cancers arising in individual organs: $CD34^+CD38^-$ for acute myeloid leukemia, $CD44^+CD24^{-/low}ESA$ (epithelial-specific antigen)$^+$ for breast cancer, $CD44^+CD24^+ESA^+$ for pancreas cancer, $CD44^+$integrin $\alpha2/\beta1^+$ for prostate cancer, $CD133^+$ for brain tumor and colorectal cancer, etc. [1,6,26,36,54]. In gastric cancer, although definitely specific markers have not been identified, CD44 surely marks cancer stem cell populations [47]. CD44-positive gastric cancer cells show stem cell properties of self-renewal and have the ability to raise CD-negative cells. CD44 knockdown results in reduced spheroid colony formation, which is characteristic of cancer stem cells, and less tumor production in immunodeficient mice. The SP cells of gastric cancer strongly express CD44, integrin $\alpha2$, $\alpha5$, $\beta3$, $\beta5$, and known stemness markers, such as Oct3/4 and Sox2 [33]. It has been reported that other potential cancer stem cell markers, such as CD24, CD133, CD166, stage-specific embryonic antigen-1 (SSEA-1), and SSEA-4, did not show any correlation with tumorigenicity [47].

Cancer stem cells share a self-renewal feature of normal tissue stem cells. Clark and Fuller [10] described that there are two ways for tumors containing a subpopulation of cancer stem cells. In the first, oncogenic mutations may inactivate the constraints on normal stem cell expansion, resulting in cancer stem cells originating from normal stem cells. The difference between normal stem cells and cancer stem cells is suggested to lie in their degree of dependence on the stem cell niche, a specialized microenvironment where stem cells reside [27]. In the second, oncogenic mutations may arise that allow for aberrant activation of the stem cell self-renewal regulatory machinery in transit-amplifying cells [10]. In the intestinal carcinogenesis, specific loss of adenomatous polyposis coli (APC) in long-lived intestinal stem cells marked by Lgr5 results in transformation and progressively growing neoplasia [4]. Regarding the stomach, it has been shown that stem cells marked by mitochondrial DNA mutations expand in normal mucosa and intestinal metaplasia, which gives an idea of how field cancerization develops [30]. However, whether or not gastric cancer stem cells arise from a normal stem cell or from transit-amplifying progenitor cell remains to be elucidated.

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References

[1] M. Al-Hajj, M.S. Wicha, A. Benito-Hernandez, S.J. Morrison, M.F. Clarke, Prospective identification of tumorigenic breast cancer cells, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 3983–3988.
[2] R. Almeida, E. Silva, F. Santos-Silva, D.G. Silberg, J. Wang, C. De Bolós, L. David, Expression of intestine-specific transcription factors, CDX1 and CDX2, in intestinal metaplasia and gastric carcinomas, J. Pathol. 199 (2003) 36–40.
[3] T. Ando, T. Yoshida, S. Enomoto, K. Asada, M. Tatematsu, M. Ichinose, T. Sugiyama, T. Ushijima, DNA methylation of microRNA genes in gastric mucosa of gastric cancer patients: its possible involvement in the formation of epigenetic field defect, Int. J. Cancer 124 (2009) 2367–2374.
[4] N. Barker, R.A. Ridgway, J.H. van Es, M. van de Wetering, H. Begthel, M. van den Born, E. Danenberg, A.R. Clarke, O.J. Sansom, H. Clevers, Crypt stem cells as the cells-of-origin of intestinal cancer, Nature 457 (2009) 608–611.
[5] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell 116 (2004) 281–297.
[6] D. Bonnet, J.E. Dick, Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell, Nat. Med. 3 (1997) 730–737.
[7] C.P. Bracken, P.A. Gregory, N. Kolesnikoff, A.G. Bert, J. Wang, M.F. Shannon, G.J. Goodall, A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial–mesenchymal transition, Cancer Res. 68 (2008) 7846–7854.
[8] G.A. Calin, C.M. Croce, MicroRNA-cancer connection: the beginning of a new tale, Cancer Res. 66 (2006) 7390–7394.
[9] M.F. Clarke, J.E. Dick, P.B. Dirks, C.J. Eaves, C.H. Jamieson, D.L. Jones, J. Visvader, I.L. Weissman, G.M. Wahl, Cancer stem cells—perspectives on current status and future directions: AACR workshop on cancer stem cells, Cancer Res. 66 (2006) 9339–9344.
[10] M.F. Clarke, M. Fuller, Stem cells and cancer: two faces of eve, Cell 124 (2006) 1111–1115.
[11] A.H. Dantzig, J.A. Hoskins, L.B. Tabas, S. Bright, R.L. Shepard, I.L. Jenkins, D.C. Duckworth, J.R. Sportsman, D. Mackensen, P.R. Rosteck Jr., P.L. Skatrud, Association of intestinal peptide transport with a protein related to the cadherin superfamily, Science 264 (1994) 430–433.
[12] E.M. El-Omar, M. Carrington, W.H. Chow, K.E. McColl, J.H. Bream, H.A. Young, J. Herrera, J. Lissowska, C.C. Yuan, N. Rothman, G. Lanyon, M. Martin, J.F. Fraumeni Jr., C.S. Rabkin, Interleukin-1 polymorphisms associated with increased risk of gastric cancer, Nature 404 (2000) 398–402.
[13] K. Fukuda, Y. Saikawa, M. Ohashi, K. Kumagai, M. Kitajima, H. Okano, Y. Matsuzaki, Y. Kitagawa, Tumor initiating potential of side population cells in human gastric cancer, Int. J. Oncol. 34 (2009) 1201–1207.
[14] C.A. Gonzalez, N. Sala, G. Capella, Genetic susceptibility and gastric cancer risk, Int. J. Cancer 100 (2002) 249–260.
[15] Y. Goto, T. Ando, K. Yamamoto, A. Tamakoshi, E. El-Omar, H. Goto, N. Hamajima, Association between serum pepsinogens and polymorphism of PTPN11 encoding SHP-2 among *Helicobacter pylori* seropositive Japanese, Int. J. Cancer 118 (2006) 203–208.
[16] N. Hamajima, M. Naito, T. Kondo, Y. Goto, Genetic factors involved in the development of *Helicobacter pylori*-related gastric cancer, Cancer Sci. 97 (2006) 1129–1138.
[17] N. Haraguchi, T. Utsunomiya, H. Inoue, F. Tanaka, K. Mimori, G.F. Barnard, M. Mori, Characterization of a side population of cancer cells from human gastrointestinal system, Stem Cells. 24 (2006) 506–513.
[18] J.C. Hartupee, H. Zhang, M.F. Bonaldo, M.B. Soares, B.K. Dieckgraefe, Isolation and characterization of a cDNA encoding a novel member of the human regenerating protein family: Reg IV, Biochim. Biophys. Acta 1518 (2001) 287–293.
[19] M. Hatakeyama, Linking epithelial polarity and carcinogenesis by multitasking *Helicobacter pylori* virulence factor CagA, Oncogene 27 (2008) 7047–7054.
[20] F. Innocenti, S.D. Undevia, L. Iyer, P.X. Chen, S. Das, M. Kocherginsky, T. Karrison, L. Janisch, J. Ramírez, C.M. Rudin, E.E. Vokes, M.J. Ratain, Genetic variants in the *UDP-glucuronosyltransferase 1A1* gene predict the risk of severe neutropenia of irinotecan, J. Clin. Oncol. 22 (2004) 1382–1388.
[21] C.T. Jordan, M.L. Guzman, M. Noble, Cancer stem cells, N. Engl. J. Med. 355 (2006) 1253–1261.
[22] M. Korpal, E.S. Lee, Y. Kang, The miR-200 family inhibits epithelial–mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2, J. Biol. Chem. 283 (2008) 14910–14914.
[23] K. Kuraoka, N. Oue, S. Matsumura, Y. Hamai, R. Ito, H. Nakayama, W. Yasui, A single nucleotide polymorphism in the transmembrane domain coding region of HER-2 is associated with development and malignant phenotype of gastric cancer, Int. J. Cancer 107 (2003) 593–596.
[24] R. Kushima, W. Muller, M. Stolte, F. Borchad, Differential p53 protein expression in stomach adenomas of gastric and intestinal phenotypes: possible sequences of p53 alteration in stomach carcinogenesis, Virchows Arch. 428 (1996) 223–227.
[25] H.J. Lawrence, C. Largman, Homeobox genes in normal hematopoiesis and leukemia, Blood 80 (1992) 2445–2453.
[26] C. Li, D.G. Heidt, P. Dalerba, C.F. Burant, L. Zhang, V. Adsay, M. Wicha, M.F. Clarke, D.M. Simeone, Identification of pancreatic cancer stem cells, Cancer Res. 67 (2007) 1030–1037.
[27] L. Li, W.B. Neaves, Normal stem cells and cancer stem cells: the niche matters, Cancer Res. 66 (2006) 4553–4557.
[28] W. Liu, L. Chen, J. Zhu, G.P. Rodgers, The glycoprotein hGC-1 binds to cadherin and lectins, Exp. Cell Res. 312 (2006) 1785–1797.
[29] S. Matsumura, N. Oue, H. Nakayama, Y. Kitadai, K. Yoshida, Y. Yamaguchi, K. Imai, K. Nakachi, K. Matsusaki, K. Chayama, W. Yasui, A single nucleotide polymorphism of the MMP9 promoter affects tumor progression and invasive phenotype of gastric cancer, J. Cancer Res. Clin. Oncol. 131 (2005) 19–25.
[30] S.A. McDonald, L.C. Greaves, L. Gutierrez-Gonzalez, M. Rodriguez-Justo, M. Deheragoda, S.J. Leedham, R.W. Taylor, C.Y. Lee, S.L. Preston, M. Lovell, T. Hunt, G. Elia, D. Oukrif, R. Harrison, M.R. Novelli, I. Mitchell, D.L. Stoker, D.M. Turnbull, J.A. Jankowski, N.A. Wright, Mechanisms of field cancerization in the human stomach: the expansion and spread of mutated gastric stem cells, Gastroenterology 134 (2008) 500–510.
[31] Y. Mitani, N. Oue, S. Matsumura, K. Yoshida, T. Noguchi, M. Ito, S. Tanaka, H. Kuniyasu, N. Kamata, W. Yasui, Reg IV is a serum biomarker for gastric cancer patients and predicts response to 5-fluorouracil-based chemotherapy, Oncogene 26 (2007) 4383–4393.

[32] T. Niimi, K. Nagashima, J.M. Ward, P. Minoo, D.B. Zimonjic, N.C. Popescu, S. Kimura, claudin-18, a novel downstream target gene for the T/EBP/NKX2.1 homeodomain transcription factor, encodes lung- and stomach-specific isoforms through alternative splicing, Mol. Cell. Biol. 21 (2001) 7380–7390.
[33] T. Nishii, M. Yashiro, O. Shinto, T. Sawada, M. Ohira, K. Hirakawa, Cancer stem cell-like SP cells have a high adhesion ability to the peritoneum in gastric carcinoma, Cancer Sci. 100 (2009) 1397–1402.
[34] N. Oue, Y. Hamai, Y. Mitani, S. Matsumura, Y. Oshimo, P.P. Aung, K. Kuraoka, H. Nakayama, W. Yasui, 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 (2004) 2397–2405.
[35] N. Oue, K. Sentani, T. Noguchi, S. Ohara, N. Sakamoto, T. Hayashi, K. Anami, J. Motoshita, M. Ito, S. Tanaka, K. Yoshida, W. Yasui, Serum olfactomedin 4 (GW112, hGC-1) in combination with Reg IV is a highly sensitive biomarker for gastric cancer patients, Int. J. Cancer 125 (2009) 2383–2392.
[36] L. Patrawala, T. Calhoun-Davis, R. Schneider-Broussard, D.G. Tang, Hierarchical organization of prostate cancer cells in xenograft tumors: the CD44+alpha2beta1+ cell population is enriched in tumor-initiating cells, Cancer Res. 67 (2007) 6796–6805.
[37] F. Petrocca, R. Visone, M.R. Onelli, M.H. Shah, M.S. Nicoloso, I. de Martino, D. Iliopoulos, E. Pilozzi, C.G. Liu, M. Negrini, L. Cavazzini, S. Volinia, H. Alder, L.P. Ruco, G. Baldassarre, C.M. Croce, A.A. Vecchione, E2F1-regulated microRNAs impair TGFβ-dependent cell-cycle arrest and apoptosis in gastric cancer, Cancer Cell 13 (2008) 272–286.
[38] Y. Sanada, N. Oue, Y. Mitani, K. Yoshida, H. Nakayama, W. Yasui, Down-regulation of the claudin-18 gene, identified through serial analysis of gene expression data analysis, in gastric cancer with an intestinal phenotype, J. Pathol. 208 (2006) 633–642.
[39] G.K. Scott, M.D. Mattie, C.E. Berger, S.C. Benz, C.C. Benz, Rapit alteration of microRNA levels by histone deacetylase inhibition, Cancer Res. 66 (2006) 1277–1281.
[40] G.E. Schepers, R.D. Teasdale, P. Koopman, Twenty pairs of sox: extent, homology, and nomenclature of the mouse and human sox transcription factor gene families, Dev. Cell 3 (2002) 167–170.
[41] N. Shibata, J. Watari, M. Fujiya, S. Tanno, Y. Saitoh, Y. Kohgo, Cell kinetics and genetic instabilities in differentiated type early gastric cancers with different mucin phenotype, Hum. Pathol. 34 (2003) 32–40.
[42] R. Spizzo, M.S. NIcoloso, C.M. Croce, G.A. Calin, SnapShot: microRNAs in cancer, Cell 137 (2009) 586.
[43] T. Suzuki, K. Matsuo, A. Sawaki, K. Wakai, K. Hirose, H. Ito, T. Saito, T. Nakamura, K. Yamao, N. Hamajima, K. Tajima, Influence of smoking and CYP2C19 genotype on *H. pylori* eradication success, Epidemiol. Infect. 135 (2007) 171–176.
[44] T. Suzuki, W. Yasui, H. Yokozaki, K. Naka, T. Ishikawa, E. Tahara, Expression of the E2F family in human gastrointestinal carcinomas, Int. J. Cancer 81 (1999) 535–538.
[45] Y. Tajima, T. Shimoda, Y. Nakanishi, N. Yokoyama, T. Tanaka, K. Shimizu, T. Saito, M. Kawamura, M. Kusano, K. Kumagai, Gastric and intestinal phenotypic marker expression in gastric carcinomas and its prognostic significance: immunohistochemical analysis of 136 lesions, Oncology 61 (2001) 212–220.
[46] S. Takaishi, T. Okumura, T.C. Wang, Gastric cancer stem cells, J. Clin. Oncol. 26 (2008) 2876–2882.
[47] S. Takaishi, T. Okumura, S. Tu, S.S. Wang, W. Shibata, R. Vigneshwaran, S.A. Gordon, Y. Shimada, T.C. Wang, Identification of gastric cancer stem cells using the cell surface marker CD44, Stem Cells 27 (2009) 1006–1020.
[48] M. Tatematsu, T. Tsukamoto, T. Mizoshita, History of gastric carcinoma research in Japan: basic aspects, in: M. Kaminishi, K. Takubo, K. Mafune (Eds.), The Diversity of Gastric Carcinoma, Springer-Verlag, Tokyo, 2005, pp. 3–28.
[49] The Study Group of Millennium Genome Project for Cancer, in: Genetic variation in PSCA is associated with susceptibility to diffuse-type gastric cancer, Nat. Genet. 40 (2008) 730–740.
[50] T. Tsukamoto, T. Mizoshita, M. Mihara, H. Tanaka, Y. Takenaka, Y. Yamamura, S. Nakamura, T. Ushijima, M. Tatematsu, Sox2 expression in human stomach adenocarcinomas with gastric and gastric-and-intestinal-mixed phenotypes, Histopathology 46 (2005) 649–658.
[51] S. Tsukita, M. Furuse, M. Itoh, Multifunctional strands in tight junctions, Nat. Rev. Mol. Cell. Biol. 2 (2001) 285–293.
[52] T. Ueda, S. Volinia, H. Okumura, M. Shimizu, C. Taccioli, S. Rossi, H. Alder, C.-G. Liu, N. Oue, W. Yasui, K. Yoshida, H. Sasaki, S. Nomura, Y. Seto, M. Kaminishi, G. Calin, C.M. Carlo, Relation between microRNA expression and progression and prognosis of gastric cancer: a microRNA expression analysis, Lancet Oncol. 11 (2010) 136–146.
[53] L.G. van der Flier, A. Haegebarth, D.E. Stange, M. van der Wetering, G. Clevers, OLFM4 is a robust marker for stem cell in human intestine and marks a subset of colorectal cancer cells, Gastroenterology 137 (2009) 15–17.
[54] L. Vermeulen, M. Todaro, F. de Sousa Mello, M.R. Sprick, K. Kemper, M. Perez Alea, D.J. Richel, G. Stassi, J.P. Medema, Single-cell cloning of colon cancer stem cells reveals a multi-lineage differentiation capacity, Proc. Natl. Acad. Sci. U.S.A. 105 (2008) 13427–13432.
[55] J.W. Watters, H.L. McLeod, Cancer pharmacogenomics: current and future applications, Biochim. Biophys. Acta 1603 (2003) 99–111.
[56] W. Yasui, N. Oue, P.P. Aung, S. Matsumura, M. Shutoh, H. Nakayama, Molecular-pathological prognostic factors of gastric cancer: a review, Gastric Cancer 8 (2005) 86–94.
[57] W. Yasui, N. Oue, R. Ito, K. Kuraoka, H. Nakayama, Search for new biomarkers of gastric cancer through serial analysis of gene expression and its clinical implications, Cancer Sci. 95 (2004) 385–392.
[58] W. Yasui, N. Oue, Y. Kitadai, H. Nakayama, Recent advances in molecular pathobiology of gastric carcinoma, in: M. Kaminishi, K. Takubo, K. Mafune (Eds.), The Diversity of Gastric Carcinoma, Springer-Verlag, Tokyo, 2005, pp. 51–71.
[59] W. Yasui, N. Oue, H. Kuniyasu, R. Ito, E. Tahara, H. Yokozaki, Molecular diagnosis of gastric cancer: present and future, Gastric Cancer 4 (2001) 113–121.
[60] W. Yasui, N. Oue, S. Ono, Y. Mitani, R. Ito, H. Nakayama, Histone acetylation and gastrointestinal carcinogenesis, Ann. N. Y. Acad. Sci. 983 (2003) 220–231.
[61] W. Yasui, N. Oue, K. Sentani, N. Sakamoto, J. Motoshita, Transcriptome dissection of gastric cancer: identification of novel diagnostic and therapeutic targets from pathology specimens (review article), Pathol. Int. 59 (2009) 121–136.
[62] W. Yasui, K. Sentani, J. Motoshita, H. Nakayama, Molecular pathobiology of gastric cancer, Scand. J. Surg. 95 (2006) 225–231.
[63] X. Zhang, Q. Huang, Z. Yang, Y. Li, C.Y. Li, GW112, a novel antiapoptotic protein that promotes tumor growth, Cancer Res. 64 (2004) 2474–2481.
[64] J. Zhang, W.L. Liu, D.C. Tang, L. Chen, M. Wang, S.D. Pack, Z. Zhuang, G.P. Rodgers, Identification and characterization of a novel member of olfactomedin-related protein family, hGC-1, expressed during myeloid lineage development, Gene 283 (2002) 83–93.

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Desmocollin 2 is a new immunohistochemical marker indicative of squamous differentiation in urothelial carcinoma

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Desmocollin 2 is a new immunohistochemical marker indicative of squamous differentiation in urothelial carcinoma

Aims: Urothelial carcinoma (UC) with squamous differentiation tends to present at higher stages than pure UC. To distinguish UC with squamous differentiation from pure UC, a sensitive and specific marker is needed. Desmocollin 2 (DSC2) is a protein localized in desmosomal junctions of stratified epithelium, but little is known about its biological significance in bladder cancer. We examined the utility of DSC2 as a diagnostic marker.

Methods and results: We analysed the immunohistochemical characteristics of DSC2, and studied the relationship of DSC2 expression with the expression of the known markers uroplakin III (UPIII), cytokeratin (CK)7, CK20, epidermal growth factor receptor (EGFR), and p53. DSC2 staining was detected in 24 of 25 (96%) cases of UC with squamous differentiation, but in none of 85 (0%) cases of pure UC. DSC2 staining was detected only in areas of squamous differentiation. DSC2 expression was mutually exclusive of UPIII expression, and was correlated with EGFR expression. Furthermore, DSC2 expression was correlated with higher stage ($P = 0.0314$) and poor prognosis ($P = 0.0477$).

Conclusions: DSC2 staining offers high sensitivity (96%) and high specificity (100%) for the detection of squamous differentiation in UC. DSC2 is a useful immunohistochemical marker for separation of UC with squamous differentiation from pure UC.

Keywords: bladder cancer, desmocollin 2, epidermal growth factor receptor, prognosis, uroplakin III, urothelial carcinoma with squamous differentiation

Abbreviations: CK, cytokeratin; DSC, desmocollin; DSG, desmoglein; EGFR, epidermal growth factor receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RNAi, RNA interference; SCC, squamous cell carcinoma; siRNA, small interfering RNA; UC, urothelial carcinoma; UPIII, uroplakin III

Introduction

Urothelial carcinoma (UC) includes the majority of bladder cancers. However, UC has a propensity for divergent differentiation. UC with squamous differentiation, which is the most common mixed histological feature, occurs in 10–60% of UC cases.[1–4] Although the clinical significance of UC with squamous differentiation

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

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

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

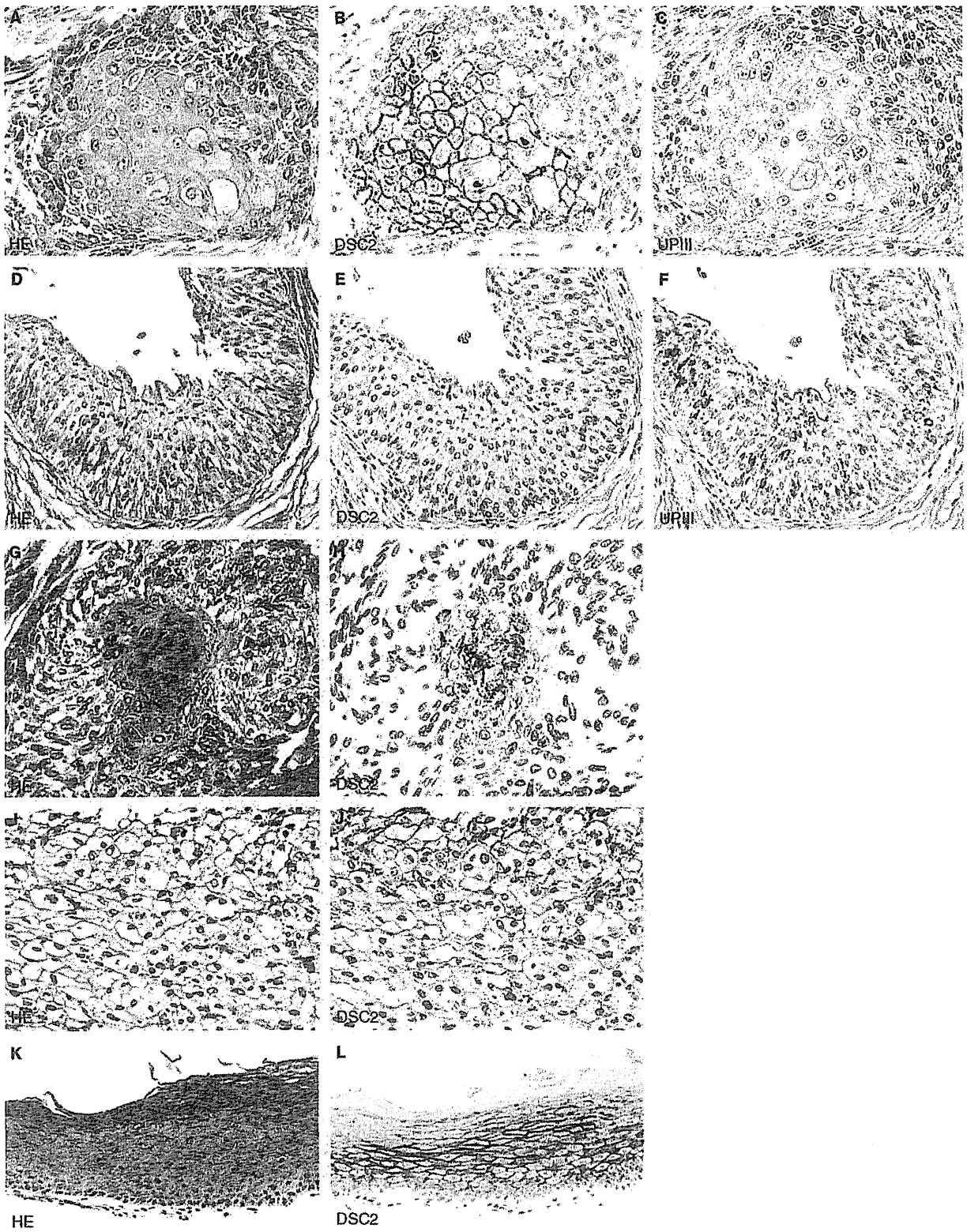
Several molecular markers appear to have some promising correlations with bladder cancer development. Among them, increased expression of epidermal growth factor receptor (EGFR) and p53 was reported to be significantly associated with high stage and poor prognosis.[27–31] Therefore, we examined the relationship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

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

Materials and methods

TISSUE SAMPLES

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



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

IMMUNOHISTOCHEMISTRY

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

CK7/20 PHENOTYPE OF UC

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

WESTERN BLOT AND CELL LINES

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

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

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

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

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

STATISTICAL METHODS

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

Results

IMMUNOHISTOCHEMICAL STAINING IN BLADDER CANCER TISSUES

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

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

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

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

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

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

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

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

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

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

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

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

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

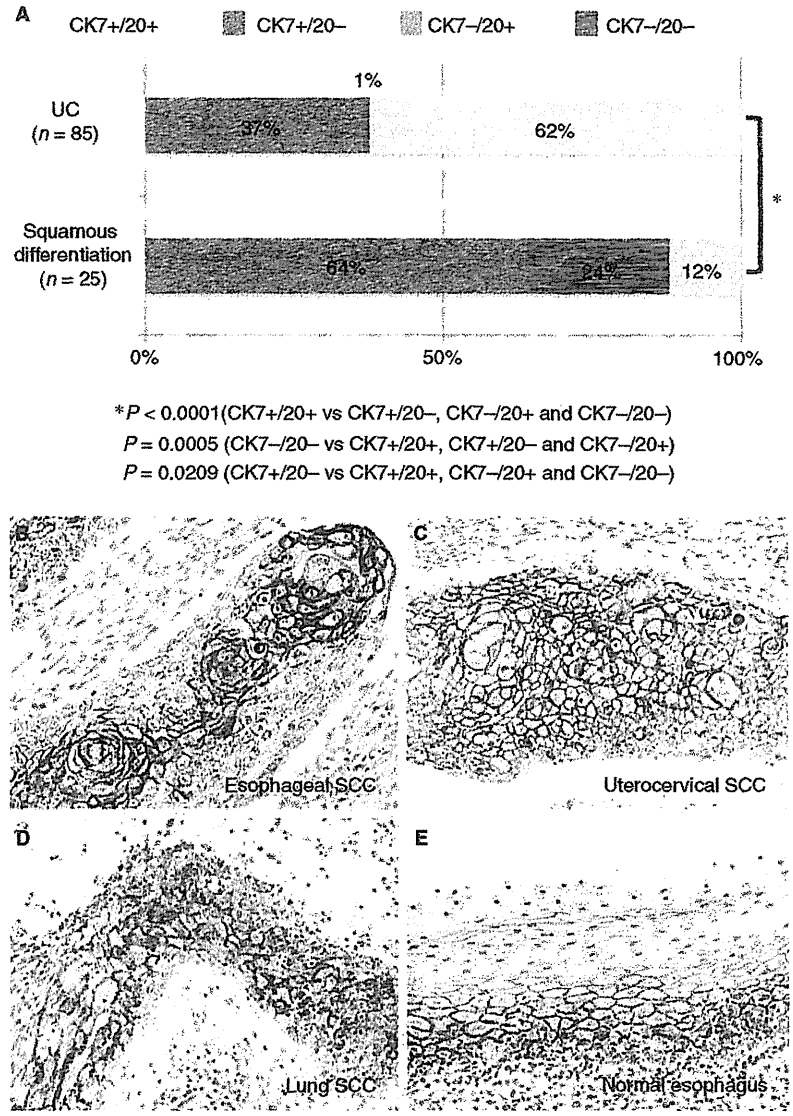


Figure 2. **A**, Summary of cytokeratin (CK)7/20 patterns compared between the area of squamous differentiation in 25 cases of urothelial carcinoma (UC) with squamous differentiation and 85 cases of pure UC. The CK7+/CK20– pattern was the predominant pattern in areas of squamous differentiation, and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

IMMUNOHISTOCHEMICAL ANALYSES IN SYSTEMIC SCC

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

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

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

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

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

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

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

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

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

RELATIONSHIP BETWEEN EXPRESSION OF DSC2 AND EGFR

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

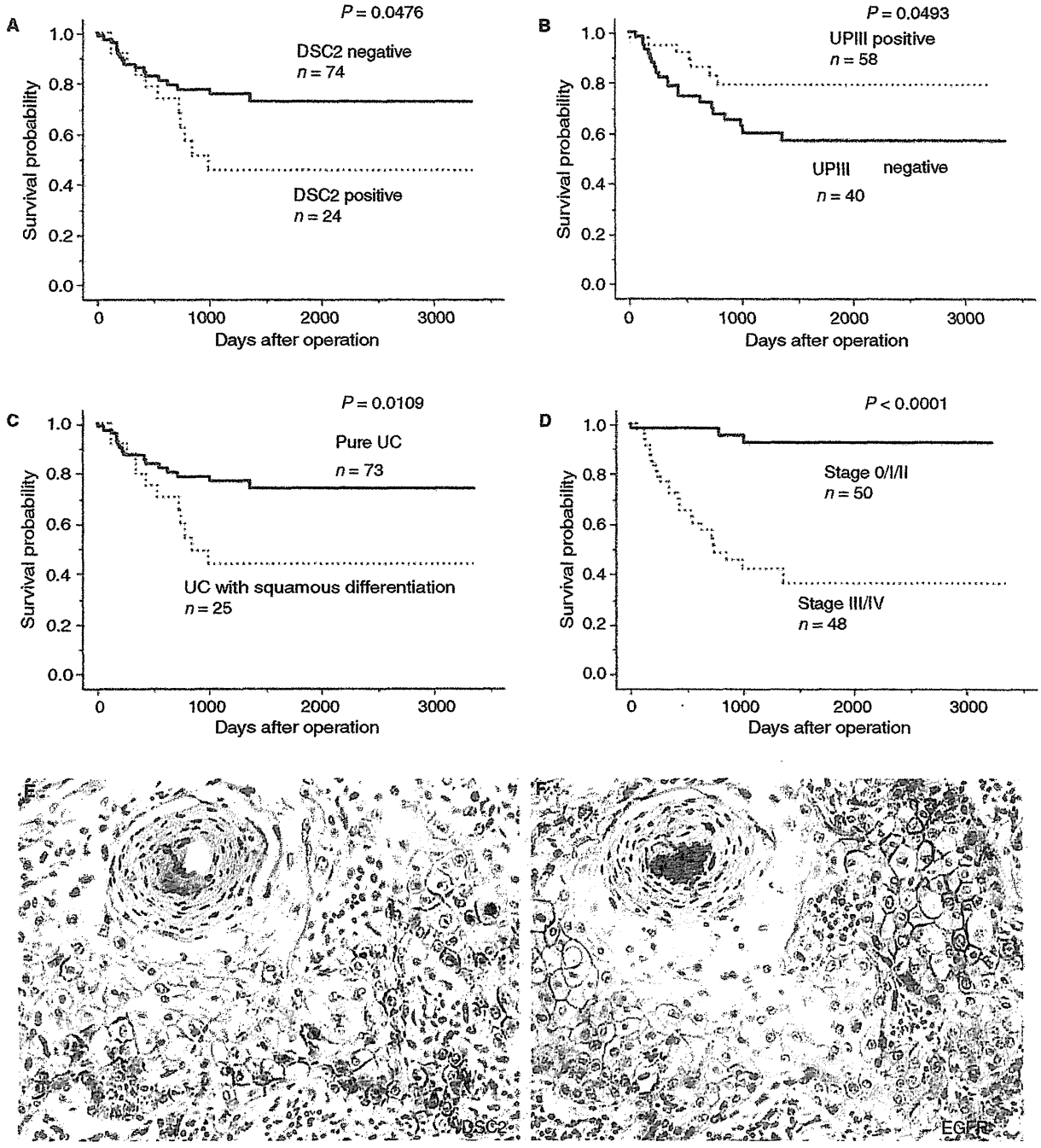


Figure 3. Prognostic values of desmocollin (DSC)2 staining (A), uroplakin III (UPIII) staining (B), histological classification (C) and stage grouping (D) in bladder cancer in 98 patients are shown by Kaplan–Meier curves. Survival of patients who were DSC2-positive or UPIII-negative, had urothelial carcinoma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

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

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

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

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

Discussion

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

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

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

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

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

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

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References

1. Martin JE, Jenkins BJ, Zuk RJ *et al.* Clinical importance of squamous metaplasia in invasive transitional cell carcinoma of the bladder. *J. Clin. Pathol.* 1989; **42**; 250–253.
2. Antunes AA, Nesrallah LJ, Dall'Oglio MF *et al.* The role of squamous differentiation in patients with transitional cell carcinoma of the bladder treated with radical cystectomy. *Int. Braz. J. Urol.* 2007; **33**; 339–345.
3. Harnden P, Southgate J. Cytokeratin 14 as a marker of squamous differentiation in transitional cell carcinomas. *J. Clin. Pathol.* 1997; **50**; 1032–1033.
4. Scosyrev E, Yao J, Messing E. Urothelial carcinoma versus squamous cell carcinoma of bladder: is survival different with stage adjustment? *Urology* 2009; **73**; 822–827.
5. Erdemir F, Tunc M, Ozcan F *et al.* The effect of squamous and/or glandular differentiation on recurrence, progression and survival in urothelial carcinoma of bladder. *Int. Urol. Nephrol.* 2007; **39**; 803–807.
6. Lagwinski N, Thomas A, Stephenson AJ *et al.* Squamous cell carcinoma of the bladder: a clinicopathologic analysis of 45 cases. *Am. J. Surg. Pathol.* 2007; **31**; 1777–1787.
7. Shokeir AA. Squamous cell carcinoma of the bladder: pathology, diagnosis and treatment. *BJU Int.* 2004; **93**; 216–220.
8. Tannenbaum SI, Carson CC 3rd, Tatum A *et al.* Squamous carcinoma of urinary bladder. *Urology* 1983; **22**; 597–599.
9. Logothetis CJ, Dexeus FH, Chong C *et al.* Cisplatin, cyclophosphamide and doxorubicin chemotherapy for unresectable urothelial tumors: the M.D. Anderson experience. *J. Urol.* 1989; **141**; 33–37.
10. Kaufmann O, Fietze E, Mengs J *et al.* Value of p63 and cytokeratin 5/6 as immunohistochemical markers for the differential diagnosis of poorly differentiated and undifferentiated carcinomas. *Am. J. Clin. Pathol.* 2001; **116**; 823–830.
11. Lopez-Beltran A, Requena MJ, Alvarez-Kindelan J *et al.* Squamous differentiation in primary urothelial carcinoma of the urinary tract as seen by MAC387 immunohistochemistry. *J. Clin. Pathol.* 2007; **60**; 332–335.
12. Dusek RL, Godsel LM, Green KJ. Discriminating roles of desmosomal cadherins: beyond desmosomal adhesion. *J. Dermatol. Sci.* 2007; **45**; 7–21.
13. Shinohara M, Hiraki A, Ikebe T *et al.* Immunohistochemical study of desmosomes in oral squamous cell carcinoma: correlation with cytokeratin and E-cadherin staining, and with tumour behaviour. *J. Pathol.* 1998; **184**; 369–381.
14. Pittella F, Katsube K, Takemura T *et al.* Perinuclear and cytoplasmic distribution of desmoglein in esophageal squamous cell carcinomas. *Pathol. Res. Pract.* 2001; **197**; 85–91.
15. Chen YJ, Chang JT, Lee L *et al.* DSG3 is overexpressed in head neck cancer and is a potential molecular target for inhibition of oncogenesis. *Oncogene* 2007; **26**; 467–476.
16. Savci-Heijink CD, Kosari F, Aubry MC *et al.* The role of desmoglein-3 in the diagnosis of squamous cell carcinoma of the lung. *Am. J. Pathol.* 2009; **174**; 1629–1637.
17. Blaveri E, Simko JP, Korkola JE *et al.* Bladder cancer outcome and subtype classification by gene expression. *Clin. Cancer Res.* 2005; **11**; 4044–4055.
18. Nuber UA, Schäfer S, Schmidt A *et al.* The widespread human desmocollin Dsc2 and tissue-specific patterns of synthesis of various desmocollin subtypes. *Eur. J. Cell Biol.* 1995; **66**; 69–74.
19. Khan K, Hardy R, Haq A *et al.* Desmocollin switching in colorectal cancer. *Br. J. Cancer* 2006; **95**; 1367–1370.
20. 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.
21. Kurzen H, Münzing I, Hartschuh W. Expression of desmosomal proteins in squamous cell carcinomas of the skin. *J. Cutan. Pathol.* 2003; **30**; 621–630.
22. Fang WK, Gu W, Li EM *et al.* Reduced membranous and ectopic cytoplasmic expression of DSC2 in esophageal squamous cell carcinoma: an independent prognostic factor. *Hum. Pathol.* 2010; **41**; 1456–1465.
23. Kaufmann O, Volmerig J, Dietel M. Uroplakin III is a highly specific and moderately sensitive immunohistochemical marker for primary and metastatic urothelial carcinomas. *Am. J. Clin. Pathol.* 2000; **113**; 683–687.
24. Parker DC, Folpe AL, Bell J *et al.* Potential utility of uroplakin III, thrombomodulin, high molecular weight cytokeratin, and cytokeratin 20 in noninvasive, invasive, and metastatic urothelial (transitional cell) carcinomas. *Am. J. Surg. Pathol.* 2003; **27**; 1–10.
25. Chu P, Wu E, Weiss LM. Cytokeratin 7 and cytokeratin 20 expression in epithelial neoplasms: a survey of 435 cases. *Mod. Pathol.* 2000; **13**; 962–972.
26. Jiang J, Ulbright TM, Younger C *et al.* Cytokeratin 7 and cytokeratin 20 in primary urinary bladder carcinoma and matched lymph node metastasis. *Arch. Pathol. Lab. Med.* 2001; **125**; 921–923.
27. Neal DE, Sharples L, Smith K *et al.* The epidermal growth factor receptor and the prognosis of bladder cancer. *Cancer* 1990; **65**; 1619–1625.
28. Mellon JK, Wright C, Kelly P *et al.* Long-term outcome related to EGFR status in bladder cancer. *J. Urol.* 1995; **153**; 919–925.
29. Nguyen PL, Swanson PE, Jaszcz W *et al.* Expression of epidermal growth factor receptor in invasive transitional cell carcinoma of the urinary bladder: a multivariate survival analysis. *Am. J. Clin. Pathol.* 1994; **101**; 166–176.
30. Sarkis AS, Dalbagni G, Cordon-Cardo C *et al.* Nuclear overexpression of p53 protein in transitional cell bladder carcinoma: a marker for disease progression. *J. Natl Cancer Inst.* 1993; **85**; 53–59.
31. Lipponen PK. Over-expression of p53 nuclear oncoprotein in transitional cell bladder-cancer and its prognostic value. *Int. J. Cancer* 1993; **53**; 365–370.
32. 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.
33. Sobin LH, Wittekind CH eds. *TNM Classification of Malignant Tumors*, 6th edn. New York: Wiley-Liss, 2002.
34. 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.
35. 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.
36. 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.
37. Mantel N. Evaluation of survival data and two new rank order statistics arising in its consideration. *Cancer Chemother. Rep.* 1966; **50**; 163–170.
38. Monica V, Ceppi P, Righi L *et al.* Desmocollin-3: a new marker of squamous differentiation in undifferentiated large-cell carcinoma of the lung. *Mod. Pathol.* 2009; **22**; 709–717.
39. Harada H, Iwatsuki K, Ohtsuka M *et al.* Abnormal desmoglein expression by squamous cell carcinoma cells. *Acta Derm. Venereol.* 1996; **76**; 417–420.
40. Tot T. Cytokeratins 20 and 7 as biomarkers: usefulness in discriminating primary from metastatic adenocarcinoma. *Eur. J. Cancer* 2002; **38**; 758–763.
41. Rotterud R, Nesland JM, Berner A *et al.* Expression of the epidermal growth factor receptor family in normal and malignant urothelium. *BJU Int.* 2005; **95**; 1344–1350.
42. Nutt JE, Mellon JK, Qureshi K *et al.* Matrix metalloproteinase-1 is induced by epidermal growth factor in human bladder tumour cell lines and is detectable in the urine of patients with bladder tumours. *Br. J. Cancer* 1998; **78**; 215–220.
43. Guo CC, Fine SW, Epstein JI. Noninvasive squamous lesions in the urinary bladder: a clinicopathologic analysis of 29 cases. *Am. J. Surg. Pathol.* 2006; **30**; 883–891.
44. Guo CC, Gomez E, Tamboli P *et al.* Squamous cell carcinoma of the urinary bladder: a clinicopathologic and immunohistochemical study of 16 cases. *Hum. Pathol.* 2009; **40**; 1448–1452.

Supporting Information

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

Data S1. Materials and methods.

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

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

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

Introduction

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

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

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

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

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

Materials and Methods

Cell Lines

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

CAST Library Construction

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

Tissue Samples

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

Quantitative RT-PCR Analysis

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

Table 1. Properties of sequenced ampicillin-resistant colonies

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

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

Evaluation of the Specificity of Gene Expression

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

RNA Interference

To knockdown the endogenous *CDON*, RNA interference was performed. Small interfering RNA (siRNA) oligonucleotides for CDON and a negative control were purchased from Invitrogen. Three independent oligonucleotides were used for *CDON* siRNA. The *CDON* siRNA1 sequence was 5′-UAU GGA GAG AGC UUG CAC CAG CUU G-3′. The *CDON* siRNA2 sequence was 5′-AAC AAC GGG AUA CUU AGG GAU GCC C-3′. The *CDON* siRNA3 sequence was 5′-UUA UGC AGC CAU GAG AUA CGA GUG G-3′. Transfection was performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. Briefly, 60 pmol siRNA and 10 μl Lipofectamine RNAiMAX were mixed in 1 ml RPMI medium (10 nmol/l final siRNA concentration). After 20 min of incubation, the mixture was added to the cells and these were plated on dishes for each assay. Forty-eight hours after transfection, cells were analyzed for all experiments.

Western Blot Analysis

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

Cell Growth, Apoptosis and in vitro Invasion Assays

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

Results

Generation of CAST Libraries

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