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transcripts at the 12-hour time point (Fig. 2B). However, as expected, cycloheximide treatment blocked induction of *MDR1* protein expression in 4-OHT–treated HT-29/CDX2-ER cells (Fig. 2B).

Inhibition of CDX2 by RNA interference results in the downregulation of *MDR1* in colon cancer cells

To determine whether CDX2 is necessary for *MDR1* expression in mammalian cells, we analyzed the effect of inhibiting CDX2 expression by RNA interference in the level of *MDR1* expression. DLD-1, a CRC cell line with high endogenous CDX2 and *MDR1* expression, was used. CDX2-specific siRNAs significantly suppressed CDX2 protein expression 3 days after transfection, and expression of *MDR1* transcript was downregulated roughly 50% by CDX2 siRNAs in DLD1 compared with its levels in parental and control siRNA-treated cells (Fig. 2C). These data indicate that CDX2 is involved in maintaining *MDR1* gene expression in gastrointestinal cell lines.

The 5′-flanking region of the *MDR1* gene contains a CDX2-responsive element

To identify potential CDX2-binding sites in the *MDR1* promoter region, genomic sequences immediately 5′ to the apparent transcription start site were searched, using a consensus-binding element for the Cdx A chicken caudal-related protein (5′-A, A/T, T, A/T, A, T, A/G-3′; ref. 23) and a previously described search algorithm (24). Four candidate CDX2-binding sites were found in the −4.0-kb region upstream of the presumptive transcription initiation sites: site A (5′-ATTTATG-3′, from −3,974 to −3,980), site B (5′-TTTTATG-3′, from −3,421 to −3,427), site C (5′-TTTTATG-3′, from −1,489 to −1,495), and site D (5′-ATTTATG-3′, from −1,463 to −1,469; Fig. 3A). To assess the role of these presumptive CDX2-binding sites in regulating *MDR1* transcription, several reporter gene constructs were generated (Fig. 3A). Reporter gene constructs containing 4.0 kb of a 5′-flanking sequence (−4,003/+50) from the *MDR1* gene showed strong activity in the HT29/PGS-CDX2 cell lines (Fig. 3B).

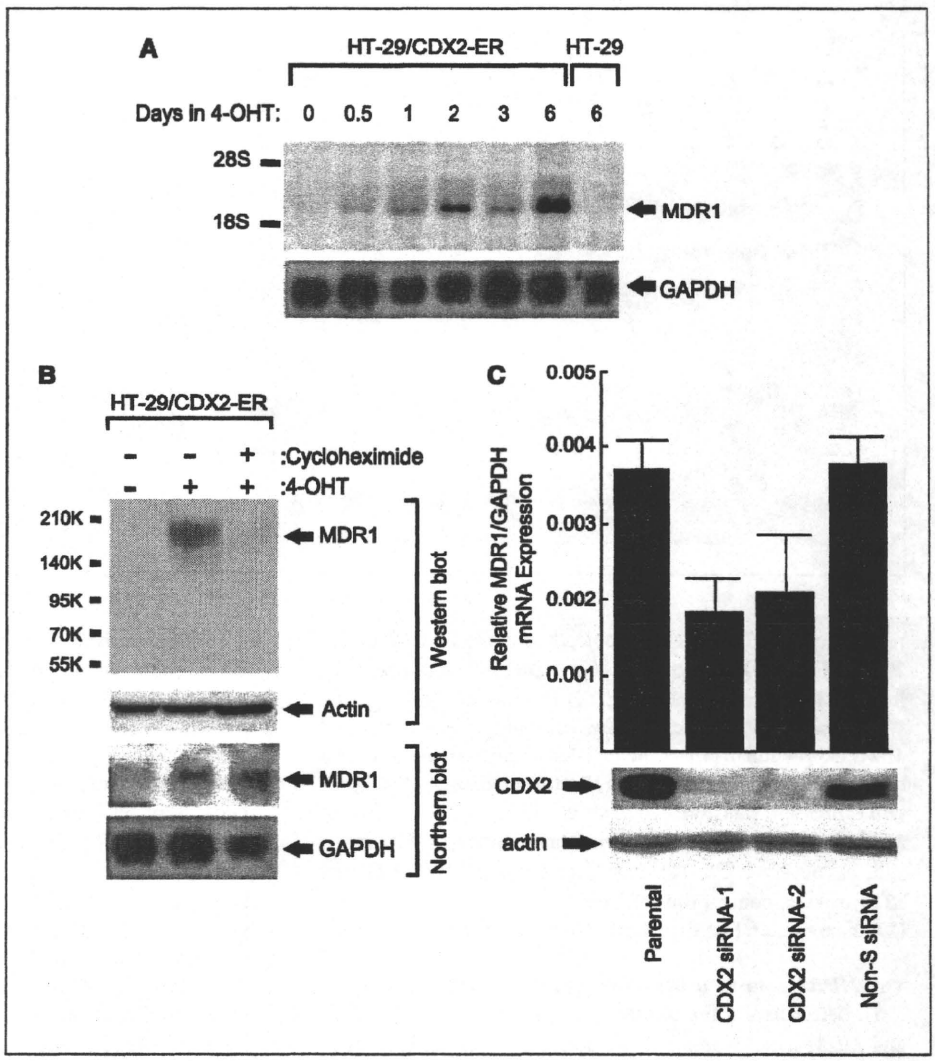

Figure 2. The *MDR1* gene is a primary target of CDX2 action. A, time course of *MDR1* gene induction in response to activation of a CDX2-ER fusion protein by 4-OHT. B, induction of *MDR1* transcript in response to activation of a CDX2-ER fusion protein by 4-OHT is not inhibited by the protein synthesis inhibitor cycloheximide, but protein synthesis is blocked. C, inhibition of CDX2 expression by siRNA targeting leads to decreased *MDR1* expression in CRC cell line DLD1. Assays were performed in triplicate; columns, mean; bars, SD.

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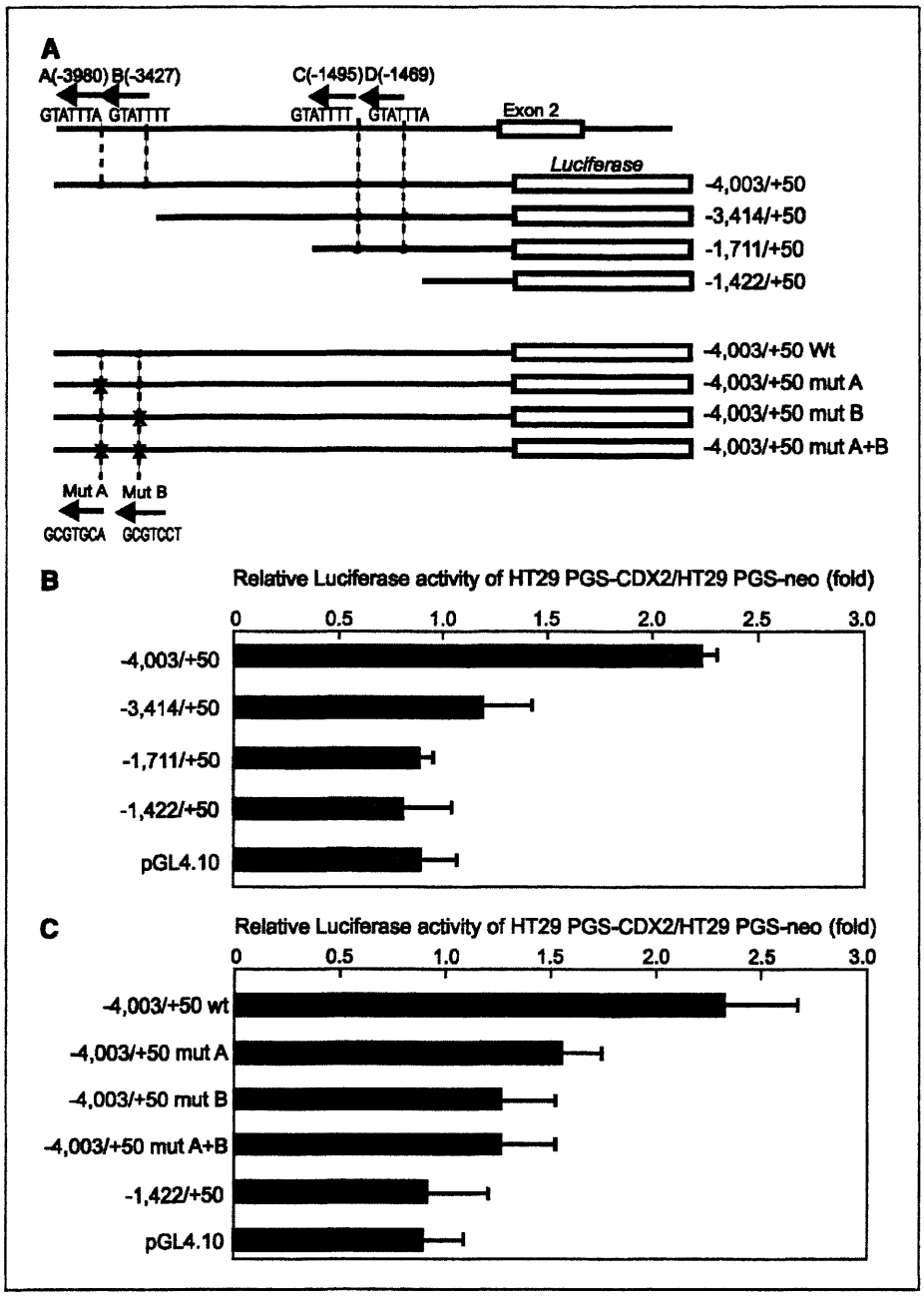

Figure 3. Localization of regulatory elements and CDX2 binding sites in the 5′-flanking region of the *MDR1* gene. A, schematic representation of the 5′-flanking region of the *MDR1* gene and *MDR1* reporter gene constructs. The location and sequence of four consensus CDX2-binding sites in the 5′-flanking region of *MDR1* are indicated. The direction of the arrows indicates the strand on which the candidate CDX2-binding element was found (i.e., sense or antisense). The *MDR1* genomic DNA sequences present in the reporter gene vectors are indicated. Localized mutations in the candidate CDX2-binding sites (i.e., site A and B) were introduced into the −4,003/+50 construct as noted (bottom), and the series of constructs generated is shown. B, key sequences for *MDR1* transcription in CDX2-expressing cell lines reside between bp −4,003 and −3,414. Columns, mean values of the luciferase activity ratio in HT29/PGS-CDX2 cells to that in HT29/PGS-neo cells; bars, SD. C, CDX2 candidate binding sites A and B play critical roles in *MDR1* transcription. All assays were performed in triplicate; columns, mean of luciferase activity ratio; bars, SD.

All the *MDR1* reporter gene constructs with deletions downstream of the 4.0-kb pair site showed decreased activity in HT29/PGS-CDX2 cell lines; thus, sequences between −3.4- and −4.0-kb pairs are important in activating *MDR1* transcription. Analysis of single and multiple mutations in the presumptive CDX2-binding sites in this region using HT29/PGS-CDX2 and HT29/PGS-neo showed that the presumptive CDX2-binding sites A and B play crucial roles in activating *MDR1* transcription (Fig. 3C).

CDX2 binds to elements in the 5′-flanking region of the *MDR1* gene

As previously noted, using the HT-29/CDX-ER cell line and the protein synthesis inhibitor cycloheximide, we found that the *MDR1* gene was a direct or primary target of CDX2. Additionally, *MDR1* reporter gene studies with localized mutations of CDX2-binding sites implied that CDX2 plays a major role in activating *MDR1* transcription by binding to one or more sites in the *MDR1* proximal promoter region. To confirm that CDX2 does indeed bind

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directly to sequences in the *MDR1* promoter region, we undertook ChIP assays using HT-29/CDX-ER cells. Before treatment of HT-29/CDX-ER cells with 4-OHT, the CDX2-ER fusion protein was expressed but remained inactive in the cells, likely because it was complexed with heat shock proteins. As would be predicted for cells lacking appreciable levels of functional CDX2, before 4-OHT treatment, we failed to recover DNA fragments of the promoter regions of *MDR1* in ChIP experiments with anti-CDX2 antibody (Fig. 4A). In contrast, on day 2 after 4-OHT–mediated activation of the CDX2-ER fusion protein, we readily recovered DNA fragments containing the *MDR1* promoter (Fig. 4A). The specificity of recovery of the *MDR1* promoter region following ChIP with anti-CDX2 antibody was shown by the fact that other irrelevant DNA fragments lacking CDX2-binding sites (e.g., exon 3 of the *CDX1* gene) were not recovered (Fig. 4A). Additionally, mock immunoprecipitation (mouse IgG whole molecule) yielded few *MDR1* or CDX1-specific DNA fragments (Fig. 4A). To confirm these data in endogenous CDX2, we performed the same ChIP assay in Caco2, CRC cell lines, which has strong endogenous CDX2 expression. We also recovered DNA fragments containing the MDR1 promoter region following ChIP with anti-CDX2 antibody (Fig. 4B). All these findings strongly suggest that CDX2 activates *MDR1* transcription by directly binding to sequences in the 5′-flanking region of the gene.

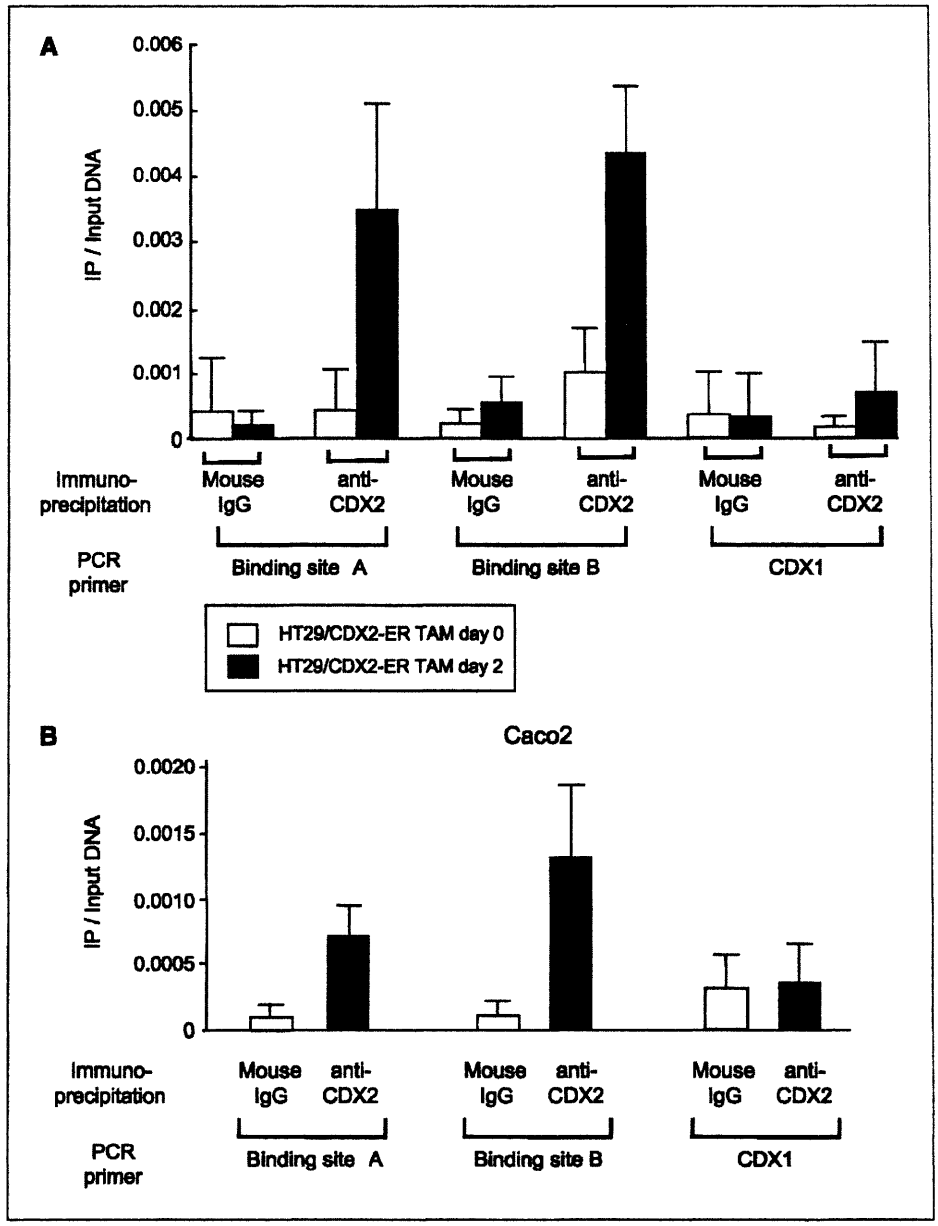

Figure 4. CDX2 binding to *MDR1* promoter region shown by ChIP. A, CDX2 function was activated in HT-29/CDX2-ER cells by treatment of the cells with 4-OHT, and the cells were harvested at the indicated time points. B, specificity of recovery of DNA fragments of MDR1 promoter region following ChIP with anti-CDX2 antibody was confirmed in Caco2, which has endogenous strong CDX2 expression. Assays were performed in triplicate, and mean and SD values are shown.

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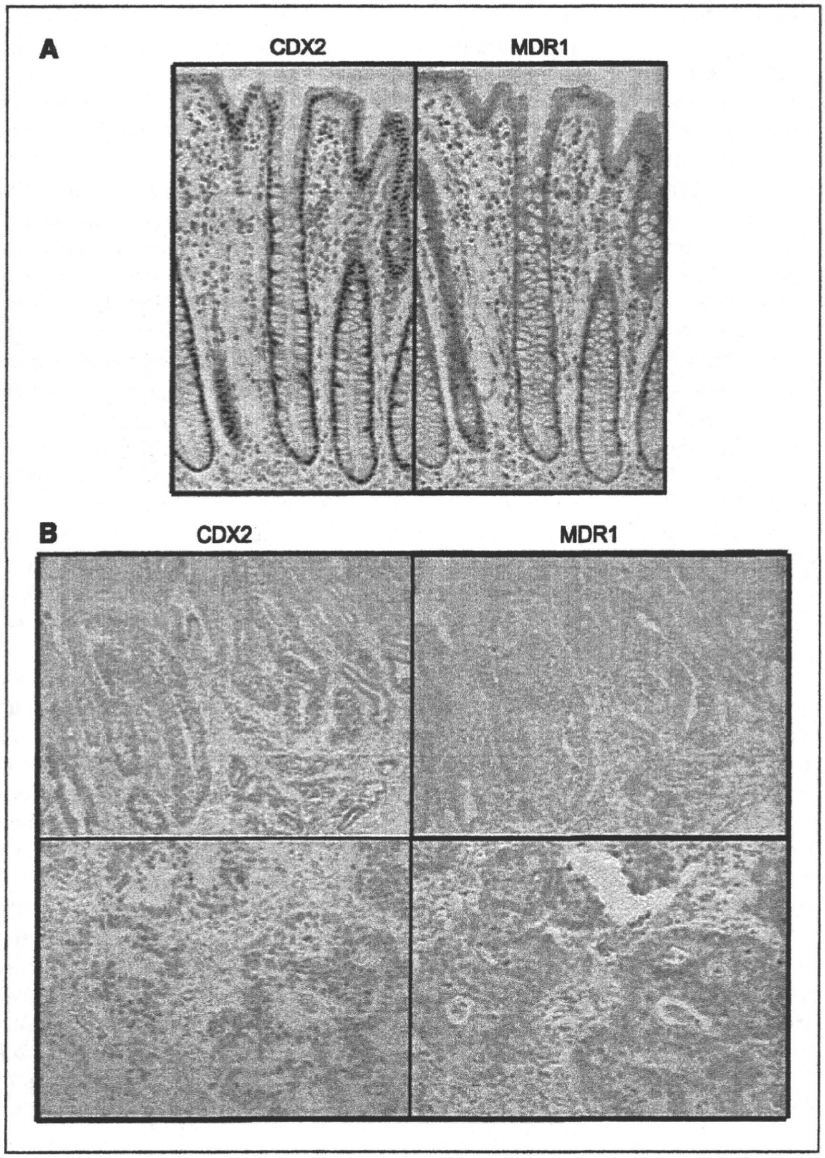

Figure 5. CDX2 and MDR1 expressions are well correlated in human colon epithelium and stomach cancer tissues. Immunohistochemistry was performed on formalin-fixed and paraffin-embedded tissues with anti-CDX2 monoclonal antibody (A and B, left) and with anti-MDR1 monoclonal antibody, C494 (A and B, right) in (A) human colon epithelium and (B) stomach cancer tissue.

CDX2 and MDR1 expression are tightly coupled in neoplastic tissues in the gastrointestinal tract

As previously noted, prior studies of CDX2 expression in normal adult tissues have shown strong CDX2 expression restricted to epithelial cells of the small intestine and colon, whereas *MDR1* is expressed in a broad range of normal tissues including epithelia of the liver; kidney; small and large intestine; and capillary endothelial cells in brain, ovary, and testis (25).

We examined the correlation between CDX2 and MDR1 expression in human healthy colon epithelium and CRC tissue microarray by immunohistochemical staining. Patterns of CDX2 and MDR1 expression are well correlated in normal colon epithelium (Fig. 5A). In CRC tissue microarray, we analyzed 302 CRC tissues. For statistical comparisons, moderate and high *MDR1* protein (P-glycoprotein) expression was evaluated against low MDR1 expression. In tissue microarray, 214 showed positive CDX2 expression (70.9%), whereas 201 showed positive MDR1 expression (66.6%). CDX2 and MDR1 expressions showed a strong positive correlation (Supplementary Table S1, $P < 0.001$). We then evaluated the correlation between CDX2 and MDR1 expression in stomach cancers because normal stomach epithelium shows low expression of both CDX2 and MDR1 (16, 26). CDX2 was stained intensely in nuclei of stomach cancer cells, whereas MDR1 was stained in the inner surface of neoplastic glands (Fig. 5B). Of 54 stomach cancers, 22 showed positive CDX2 expression (40.7%), whereas 25 showed positive MDR1 expression (46.3%). CDX2 and MDR1 expressions showed a strong positive correlation ($P < 0.001$; Supplementary Table S2).

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HT-29 cells ectopically expressing CDX2 have MDR1-dependent drug resistance

To determine whether MDR1 induced by CDX2 functions as a drug reflux pump, we analyzed the effects of chemotherapeutic drugs on HT-29/PGS-CDX2 and HT29/PGS-neo cells (Fig. 6A). The MDR1 nonsubstrates, that is, cisplatin, camptothecin, 5-fluorouracil, and doxorubicin, showed similar activity in HT-29/PGS-CDX2 and HT-29/PGS-neo cells, whereas the known MDR1 substrates (25), vincristine and paclitaxel, showed lesser activity [7.7- and 3.0-fold increase in IC_{50} (72 h), respectively] in HT-29/PGS-CDX2 cells (Fig. 6A).

To examine MDR1-dependent drug resistance, we conducted the same assay in the presence of the MDR1 inhibitor verapamil. Cotreatment with 2 μmol/L verapamil increased the activities of vincristine and paclitaxel in HT-29/PGS-CDX2 cells (Fig. 6B and C). Verapamil reduced the differences in the drug-induced cytotoxicity between HT-29/PGS-CDX2 and HT-29/PGS-neo cells (Fig. 6B and C). This suggests that increased resistance to vincristine and paclitaxel in HT-29/PGS-CDX2 cells is caused by overexpression of the *MDR1* gene.

Discussion

There is now a sizable body of data supporting the idea that the intestine-specific homeobox transcription factor CDX2 has a crucial role in directing intestinal epithelial development and differentiation (1, 2). However, the precise molecular mechanisms underlying tissue-specific expression of CDX2 and its downstream target genes remain undefined. To date, only a limited number of CDX2-regulated target genes have been suggested, including sucrase-isomaltase (27), glucagon (28), carbonic anhydrase 1 (29), calbindin-D9K (30), vitamin D receptor (31), lactase (32), guanylyl cyclase C (33), clusterin (34), gut-enriched Krüppel-like factor (35), heparin-binding epidermal growth factor–like growth factor (36), *MUC2* (37), LI-cadherin (16), *HEPH* (18), *Cdx2* itself through autoregulatory loop (38), insulin receptor substrate 2 (39), and solute carrier family 5, member 8 (SLC5A8; ref. 40).

In this study, we identified *MDR1* as a candidate gene directly regulated by CDX2. Evidence that CDX2 might regulate *MDR1* was initially obtained using high-density oligonucleotide microarrays to identify genes activated following overexpression of CDX2 in a CRC cell line showing very low endogenous CDX2 expression. Additionally, data indicating that endogenous *MDR1* expression was dependent on CDX2 were obtained, along with evidence that activation of CDX2 induced *MDR1* transcripts even in the presence of protein synthesis inhibitors. We identified four presumptive CDX2-binding sites in the 4-kb region upstream of the transcription start sites of *MDR1*. Reporter gene analysis showed that two of these elements were critical. Subsequent ChIP assays showed that CDX2 binds directly to this *MDR1* promoter region. Immunohistochemical staining analysis for 302 CRCs and 54 stomach cancers showed that CDX2 and MDR1 protein expressions were significantly correlated. Given the regulation of *MDR1* by CDX2 in neoplastic gastrointestinal tissues, CDX2, as well as MDR1, may be a useful marker for predicting the status of drug resistance in the stomach and perhaps elsewhere.

Although our data offer reliable support for the view that CDX2 plays a role in regulating *MDR1* transcription by binding to one or more elements in the proximal promoter region, CDX2 might not be sufficient for activating *MDR1* expression. It is possible that other factors along with CDX2 may be required to activate *MDR1* transcription in certain settings, such as in HT-29 cells, because two of the eight CDX2-positive CRC cell lines we studied (namely SW48 and LS174T) expressed very low or undetectable levels of *MDR1* transcripts and protein. Previously, we obtained similar evidence that CDX2 was required but not sufficient for activating *LI-cadherin* and *HEPH* transcription (16, 18). On the other hand, our data indicated that inhibition of CDX2 expression by siRNA leads to decreased *MDR1* transcription, suggesting that CDX2 does play a key role in maintaining *MDR1* expression in certain settings, such as in CDX2- and MDR1-expressing CRC cells. It will be interesting in the future to define other factors that cooperate with CDX2 in regulating *MDR1*, LI-cadherin, and HEPH expression in gastrointestinal tissues.

In our study, we showed that expression of CDX2 induced MDR1-dependent drug resistance in a CRC cell line, which was reversed by the MDR1-specific inhibitor verapamil (21), suggesting a role of CDX2 in the regulation of *MDR1* gene expression in drug resistance. Consistent with the intestine-specific expression of CDX2 in humans and mice, recent analysis for tissue-specific murine *Mdr1a* gene expression in naïve animals revealed that the basal *Mdr1a* expression level was 100-fold higher in the intestine than in other *MDR1*-expressing tissues such as the liver, kidney, and spleen (25, 41). In epithelial cells of the lower gastrointestinal tract (jejunum, ileum, and colon), high levels of MDR1 protein are found only on the apical surfaces of superficial columnar epithelial cells, which suggests a function to prevent uptake of substrates and perhaps to facilitate excretion across the mucosa of the gastrointestinal tract (26). Given the role of CDX2 in the establishment and maintenance of intestinal epithelium, CDX2 may play a critical role in protecting the intestinal epithelium and the human body from toxic xenobiotics by stably inducing *MDR1* even under naïve conditions.

In cancer tissue, the *MDR1* gene was originally identified as an overexpressed and amplified gene in multiple drug-resistant cells (19, 25). The *MDR1* gene encodes P-glycoprotein, a member of the large ATP-binding cassette superfamily of transmembrane proteins (ATP-binding cassette, sub family B, member 1) that transports structurally different hydrophobic chemotherapeutic agents outward in an energy-dependent manner. Regulation of *MDR1* gene expression is complex because like many TATA-less promoters (42), the promoter of the *MDR1* gene contains multiple start sites. In studies of CRCs, expression of *MDR1* was correlated with pathologic grading of tumors, being most intense in well-differentiated tumors and low in poorly differentiated ones (43). Similarly, moderately differentiated gastric carcinomas expressed a higher level of MDR1 than poorly differentiated

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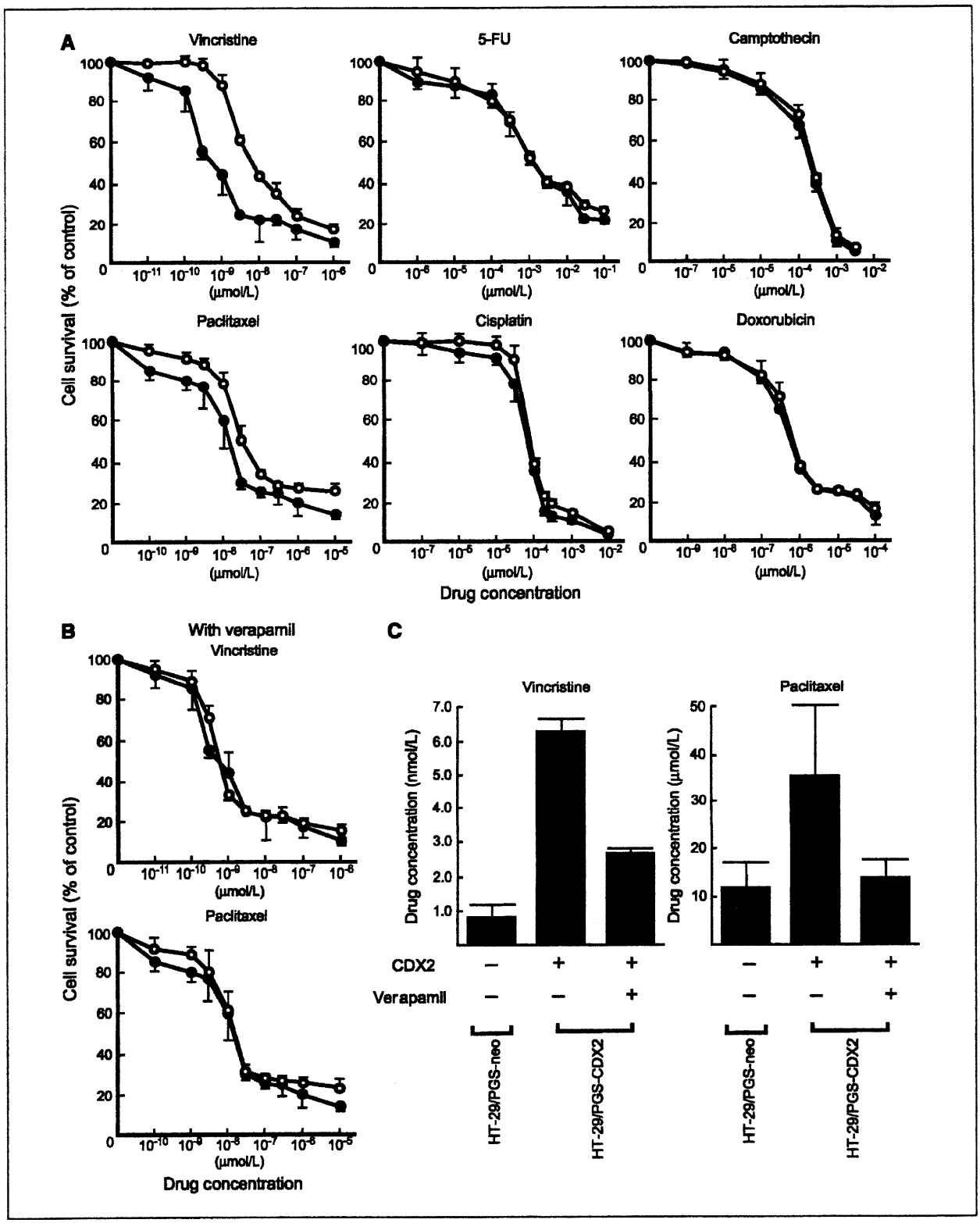

Figure 6. HT29 cells ectopically expressing CDX2 have MDR1-dependent drug resistance. A, effect of chemotherapeutic drugs on HT29/PGS-CDX2 (○) and HT29/PGS-neo (●) cell lines. B, effect of additional verapamil on vincristine and paclitaxel in HT29/PGS-CDX2 (○) and HT29/PGS-neo (●) cell lines. C, [IC_{50} (72 h)] determined by MTT assay on HT29/PGS-CDX2 and HT29/PGS-neo cells. Cotreatment with verapamil significantly recovered the sensitivity of vincristine and paclitaxel on HT-29/PGS-CDX2 cells. The cytotoxic assays were performed in triplicate; points, mean; bars, SD.

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ones (44). Although studies of CRCs arising in humans have not offered definitive proof of a causal role for CDX2 inactivation in the cancer process, it is quite clear that loss of CDX2 expression is seen in a subset of primary CRCs, particularly tumors with minimal differentiation (45). Consistent with our previous observation in large cell minimally differentiated adenocarcinoma of the colon, recent multivariate analysis also indicates that loss of CDX2 expression is associated with less-differentiated carcinoma and advanced stage, although CDX2 loss is not independently associated with patient survival (15, 46). Considering the roles of CDX2 in promoting cellular differentiation and inhibiting proliferation (45), CDX2 loss could conceivably contribute to aggressive tumor behavior, although MDR1 loss induced by CDX2 suppression may have some beneficial influence on patient survival with reduced drug resistance.

In conclusion, our findings implicating CDX2 in regulation of *MDR1* offer data on specific factors and mechanisms regulating MDR1 expression in gastrointestinal cancers. However, several outstanding issues regarding the transcriptional regulation of *MDR1* by CDX2 remain to be addressed. Due to the complexity of the mechanism of drug resistance, further studies of *MDR1* and its regulation by CDX2 in various gastrointestinal cancers should help to enhance understanding of the mechanism of aberrant (ectopic) expression of CDX2 and its downstream target *MDR1*, and in the development of a strategy to select chemotherapy regimens based on the status of CDX2 and *MDR1* expression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Upregulation of Connexin 30 in Intestinal Phenotype Gastric Cancer and Its Reduction during Tumor Progression

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Key Words
Gastric cancer · Intestinal phenotype · Connexin 30 · Microarray

Abstract
Aims: The mucin phenotype is associated with clinicopathological findings and tumorigenesis in gastric cancer (GC). The aim was to search for a novel marker regulating the intestinal phenotype of GC. ***Methods and Results:*** We performed microarray analyses, and *GJB6* (encoding connexin 30) was identified as a gene associated with the intestinal phenotype. Immunostaining of connexin 30 in 169 GC cases revealed that 47 (28%) cases were positive for connexin 30, while connexin 30 was negative in nonneoplastic gastric tissue. Connexin 30-negative GC cases showed more advanced T grade, N grade, and tumor stage than connexin 30-positive GC cases. Six (13%) GC cases positive for connexin 30 were histologically of the differentiated type. In addition, the expression of gastric and intestinal phenotypes of GC was examined by immunostaining for MUC5AC, MUC6, MUC2, and CD10. Connexin 30 expression occurred more frequently in the intestinal phenotype (48%) than in other phenotypes (21%) of GC. ***Conclusion:*** These results indicate that the expression of connexin 30 is a novel differentiation marker mediating the biological behavior of intestinal phenotype GC.

Introduction

According to the World Health Organization, gastric cancer (GC) is the fourth most common malignancy worldwide, with approximately 870,000 new cases occurring yearly. Mortality due to GC is second only to that due to lung cancer [1]. Cancer develops as a result of multiple genetic and epigenetic alterations [2, 3]. Better knowledge of the changes in gene expression that occur during gastric carcinogenesis may lead to improvements in diagnosis, treatment, and prevention. Identification of novel biomarkers for cancer diagnosis and novel targets for treatment are major goals in this field [4]. Array-based hybridization [5] and serial analysis of gene expression (SAGE) [6] are currently the most common approaches used to identify potential molecular markers for cancer.

GCs have been classified into 2 histological types: an intestinal type and a diffuse type by Lauren [7], or a differentiated type and an undifferentiated type by Nakamura et al. [8], based on the tendency toward gland formation. It has been suggested that these 2 types involve distinct pathways during carcinogenesis [7–10]. Recent studies have demonstrated that GCs are also classified as having a gastric, gastric and intestinal mixed, or intestinal phenotype depending on the expression of mucin phenotypic markers [11–18]. The mucin expression and phenotype of tumors are associated with clinicopatho-

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logical findings and tumorigenesis in GCs. However, the clinical importance of intestinal mucin in GCs is still controversial and no definite conclusions have been reached [12–18]. Candidate genes controlling gastric and intestinal phenotypes include several transcription factors [19]. The caudal-related homeobox 2 gene (CDX2) is an intestine-specific transcription factor that is expressed in nonneoplastic mucosa from the duodenum to the distal colon and is detected in GC with the intestinal phenotype [20]. SOX2, an Sry-like high-mobility group box gastric transcription factor, is expressed in normal gastric mucosa and GC with the gastric phenotype [21]. By performing microarray analyses, we recently discovered that the expression of connexin 30 was observed in intestinal phenotype GC.

Connexins, a family of 20 transmembrane proteins in humans, comprise the main subunits of gap junctions, which are specialized clusters of intercellular channels that allow adjacent cells to directly share ions and hydrophilic molecules of up to 1 kDa in size [22]. Gap junctional intercellular communication (GJIC) is thought to control tissue homeostasis and to coordinate cellular processes such as proliferation, migration, and differentiation [23, 24]. Neoplastic transformation is frequently associated with a loss of GJIC and with a reduced expression of connexins in various tumors [25, 26]. The forced expression of connexins in connexin-deficient cell lines results in the inhibition of tumor growth and the induction of apoptosis in vitro as well as the prevention of tumor formation in vivo [27, 28]. On the other hand, accumulating evidence indicates that connexin 26, a connexin family member, is overexpressed in carcinomas including those of the head and neck, colon, and pancreas [29–32]. Increased connexin 26 expression has been observed in invasive breast carcinomas and metastatic lymph nodes [33, 34]. Together, these strands of evidence appear to contradict the conventionally held view of the role of connexins as tumor suppressors. The localization of connexin 30 has been observed in normal skin [35], cochlea [36] and brain [37]. Connexin 30 gene mutations cause dominant nonsyndromic hearing loss [38, 39], and they have been identified in Clouston syndrome (hidrotic ectodermal dysplasia) [40]. Little is known about the role of connexin 30 in human neoplasia. While the expression of connexin 30 is decreased in human head and neck cancer [41] and in cervical dysplasia of the uterus [42], connexin 30 is upregulated in human skin tumors [43]. Thus, the exact pathogenic mechanisms associated with connexin 30 in carcinogenesis remain obscure.

The present study represents the first detailed analysis of connexin 30 expression in GC. To clarify the pattern of expression and localization of connexin 30 in GC, we performed immunohistochemical analysis of surgically resected GC samples. In addition, we investigated the association between connexin 30 and various markers determining the gastric/intestinal phenotypes (MUC5AC, MUC6, MUC2, and CD10).

Materials and Methods

Tissue Samples

Primary tumor samples and the corresponding nonneoplastic gastric mucosa were collected from 169 patients with GC (123 men and 46 women; age range 29–88 years; mean 70 years). Patients were treated at Hiroshima University Hospital or affiliated hospitals. For RNA extraction, tissue samples obtained at the time of surgery were immediately embedded in OCT compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), frozen in liquid nitrogen, and stored at −80°C. For quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis, 18 GC samples and the corresponding nonneoplastic mucosa samples were used. The samples were obtained during surgery at Hiroshima University Hospital. We confirmed microscopically that the tumor specimens were predominantly (>50%) cancer tissue. Samples were frozen immediately in liquid nitrogen and stored at −80°C until used. Samples of normal brain, spinal cord, heart, skeletal muscle, lung, stomach, small intestine, colon, liver, pancreas, kidney, uterus, bone marrow, spleen, peripheral leukocytes, and trachea were purchased from Clontech (Palo Alto, Calif., USA). For immunohistochemical analysis we used archival formalin-fixed paraffin-embedded tissues from 169 patients who had undergone surgical excision for GC. The 169 GC cases were histologically classified as 102 of the differentiated type and 67 of the undifferentiated type, according to the Japanese Classification of Gastric Carcinomas [44]. Tumor staging was carried out according to the TNM classification [45]. Because written informed consent was not obtained, 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.

Quantitative RT-PCR

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, Piscataway, N.J., USA). Quantitation of *Connexin 30* mRNA levels in human tissue samples was done by real-time fluorescence detection as described previously [46]. *Connexin 30* primer sequences were 5′-CAG TTG CCT TCT CTC CGA GG-3′ and 5′-CAT GGG ATG TTA CAC ACG CC-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 bound to double-stranded DNA was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as described previously [47]. *ACTB*-specific

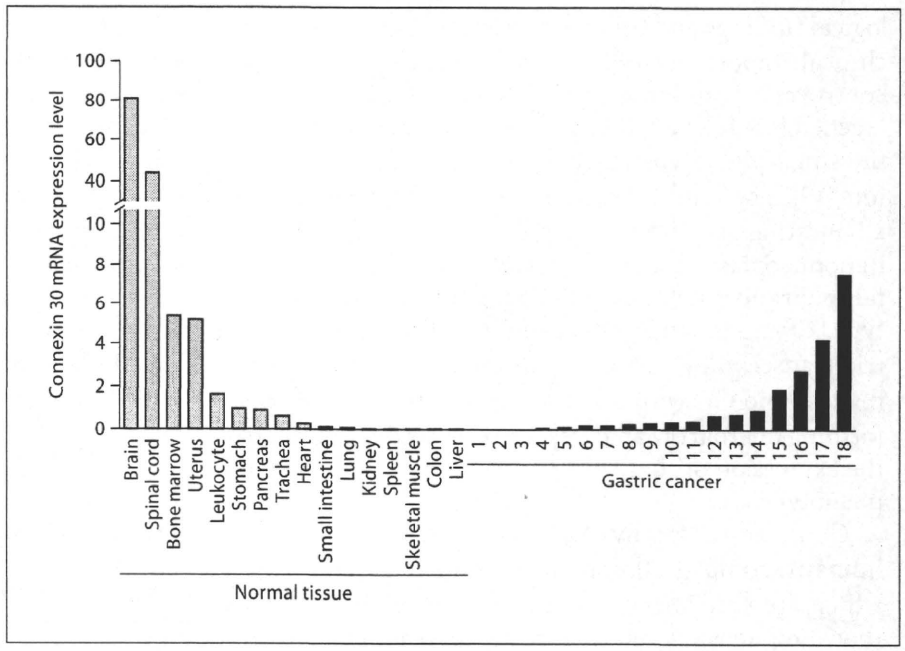

Fig. 1. Quantitative RT-PCR analysis of *connexin 30* in various human normal tissues and GC tissues. Clear *connexin 30* expression is present in normal brain, spinal cord, bone marrow, uterus, etc. High levels of *connexin 30* were observed in some GCs. The units are arbitrary and *connexin 30* expression was calculated by the standardization of 1.0 μg of total RNA from normal stomach as 1.0.

PCR products were amplified from the same RNA samples and served as internal controls.

Antibodies

Anti-connexin 30 antibody was purchased from Invitrogen/Zymed Laboratories, Inc. (San Francisco, Calif., USA). We used 4 antibodies for analysis of the GC phenotypes: anti-MUC5AC (Novocastra, Newcastle, UK) as a marker of gastric foveolar epithelial cells, anti-MUC6 (Novocastra) as a marker of pyloric gland cells, anti-MUC2 (Novocastra) as a marker of goblet cells in the small intestine and colorectum, and anti-CD10 (Novocastra) as a marker of microvilli of absorptive cells in the small intestine and colorectum.

Immunohistochemistry

A Dako LSAB Kit (Dako, Carpinteria, Calif., USA) was used for immunohistochemical analysis. In brief, sections were pretreated by microwave treatment in citrate buffer for 15 min to retrieve antigenicity. After peroxidase activity was blocked with 3% H_2O_2-methanol for 10 min, sections were incubated with normal goat serum (Dako) for 20 min to block nonspecific antibody binding sites. Sections were incubated with the following primary antibodies: anti-connexin 30, anti-MUC5AC, anti-MUC6, anti-MUC2, and anti-CD10 (all diluted 1:50). Sections were incubated with primary antibody for 1 h at 25°C, followed by incubations with biotinylated mouse anti-rabbit IgG and peroxidase-labeled streptavidin for 10 min each. Staining was completed with a 10-min incubation with the substrate-chromogen solution. The sections were counterstained with 0.1% hematoxylin.

Connexin 30 staining was classified according to the percentage of stained cancer cells. Expression was considered to be 'negative' if <10% of cancer cells were stained. When at least 10% of cancer cells were stained, the result of immunostaining was considered 'positive'.

GC cases were classified into 4 phenotypes: gastric phenotype, intestinal phenotype, gastric and intestinal mixed phenotype, and unclassified phenotype. The criteria [20] for the classification of gastric phenotype and intestinal phenotype were as follows: GCs in which more than 10% of the cells displayed the gastric or intestinal epithelial cell phenotype were gastric phenotype or intestinal phenotype cancers, respectively; those sections that showed both gastric and intestinal phenotypes were classified as gastric and intestinal mixed phenotype, and those that lacked both the gastric and the intestinal phenotypes were classified as the unclassified phenotype.

Double Immunofluorescence Staining

Double immunofluorescence staining was performed as described previously [48]. Alexa Fluor 488-conjugated chicken anti-rabbit IgG and Alexa Fluor 546-conjugated goat anti-mouse IgG were used as secondary antibodies (Molecular Probes, Eugene, Oreg., USA).

Statistical Methods

Correlations between clinicopathologic parameters and connexin 30 staining were analyzed by Fisher's exact test. $p < 0.05$ was considered statistically significant.

Results

Expression of Connexin 30 *in Systemic Normal Tissues and GC Tissues*

Quantitative RT-PCR was performed to investigate the specificity of *connexin 30* expression in 16 normal organs. As shown in figure 1, *connexin 30* expression was clearly

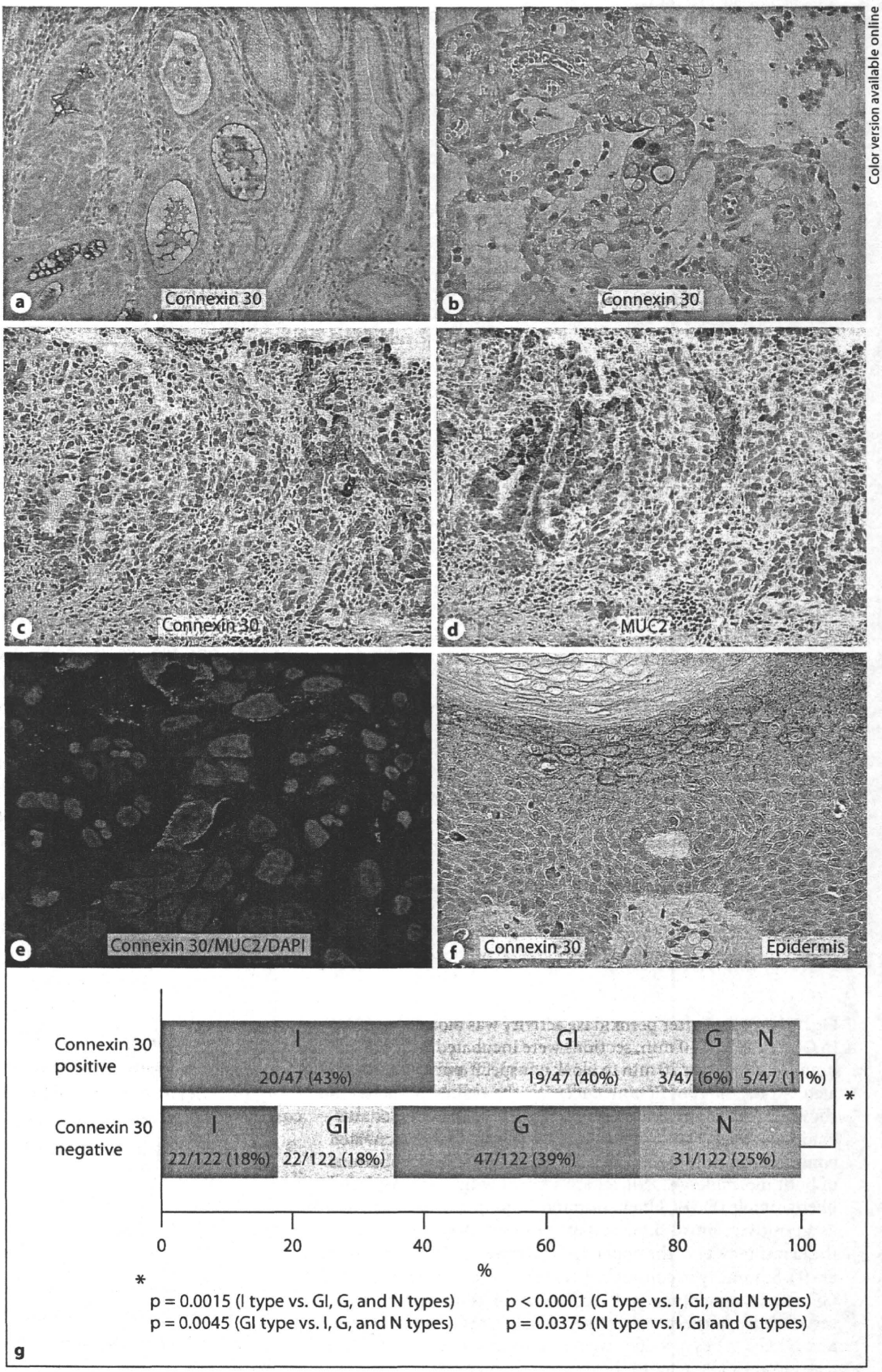
Color version available online
a
Connexin 30
b
Connexin 30
c
Connexin 30
d
MUC2
e
Connexin 30/MUC2/DAPI
f
Connexin 30
Epidermis
Connexin 30 positive
I
20/47 (43%)
GI
19/47 (40%)
G
3/47 (6%)
N
5/47 (11%)
*
Connexin 30 negative
I
22/122 (18%)
GI
22/122 (18%)
G
47/122 (39%)
N
31/122 (25%)
0
20
40
60
80
100
%
*
p = 0.0015 (I type vs. GI, G, and N types)
p = 0.0045 (GI type vs. I, G, and N types)
p < 0.0001 (G type vs. I, GI, and N types)
p = 0.0375 (N type vs. I, GI and G types)
g

Table 1. Relationship between connexin 30 expression and clinicopathologic parameters in 169 GC cases

	Connexin 30 expression		p value
	positive (n = 47)	negative (n = 122)	
Age			
≤65 years (n = 46)	13 (28)	33	NS
>65 years (n = 123)	34 (28)	89	
Gender			
Male (n = 123)	33 (27)	90	NS
Female (n = 46)	14 (30)	32	
Histology[1]			
Differentiated (n = 102)	41 (40)	61	<0.0001
Undifferentiated (n = 67)	6 (9)	61	
T grade			
T1 (n = 83)	35 (42)	48	<0.0001
T2/T3/T4 (n = 86)	12 (14)	74	
N grade			
N0 (n = 107)	37 (35)	70	0.0123
N1/N2/N3 (n = 62)	10 (16)	52	
M grade			
M0 (n = 163)	47 (29)	116	NS
M1 (n = 6)	0 (0)	6	
Stage[2]			
Stage 0/I (n = 104)	38 (37)	66	0.0014
Stage II/III/IV (n = 65)	9 (14)	56	

Figures in parentheses are percentages. p values were calculated by Fisher's exact test. NS = Not significant.

[1] Histology was classified according to the Japanese Classification of Gastric Carcinomas.

[2] Tumor stage was classified according to International Union Against Cancer TNM classification of malignant tumors criteria.

Fig. 2. Immunohistochemical staining of connexin 30 and MUC2 in GC tissues (**a–e**). Connexin 30 was detected in the apical membranes of both well-differentiated GC (**a**) and poorly differentiated GC (**b**), but not in noncancerous epithelium. Serial sections showed that expression of connexin 30 (**c**) was partly adjacent to cytoplasmic expression of MUC2 (**d**). Double-immunostaining of connexin 30 (red) and MUC2 (green) revealed no colocalization of both molecules (**e**). Nuclei are stained with 4′,6-diamidino-2-phenylindole (DAPI; blue). Immunostaining of human epidermis as a positive control showed that connexin 30 was distributed in the keratinocytes of the upper spinous layers and the granular layers (**f**). Summary of connexin 30 expression and expression of the GC mucin phenotype (**g**). Expression of connexin 30 was observed more frequently in I-type and GI-type GC than in other (G and N) GC types. p values were statistically analyzed by Fisher's exact test. Colors refer to the online version only.

detected in the brain and the spinal cord and to a lesser extent in the bone marrow and uterus. However, the expression of *connexin 30* was detected at low levels, or not at all, in other normal organs including the stomach. These results are consistent with those of a previous report [37]. Next, we analyzed quantitative RT-PCR in 18 GC samples. High levels of *connexin 30* (tumor/normal ratio >2) were observed in 4 of the 18 GCs (22%).

Immunohistochemical Analysis of Connexin 30 in GC

Quantitative RT-PCR revealed obvious *connexin 30* expression in GC, although the levels were not very high. We therefore performed immunohistochemical analysis of connexin 30 in GC (fig. 2a–e). At first, we tested the specificity of the anti-connexin 30 antibody. Immunohistochemical analysis of normal skin tissue was performed, and connexin 30 was detected in the keratinocytes of the upper spinous layers and in those of the stratum granulosum (fig. 2f). This result was consistent with a previous report [49]. Using this antibody, we performed immunostaining of connexin 30 in 169 GCs and the corresponding nonneoplastic gastric mucosa. As a result, connexin 30 expression was detected in 47 of the 169 GCs (28%) and was seen on the cell membrane, especially the apical membrane (fig. 2a, b). However, we sometimes observed its cytoplasmic accumulation (fig. 2c). There was no difference in connexin 30 expression levels between intratumor areas and infiltrative margins.

Connexin 30 was scarcely expressed in any corresponding nonneoplastic gastric mucosa or intestinal metaplasia. Next, we analyzed the relationship between connexin 30 expression and clinicopathologic characteristics. The expression of connexin 30 was observed more frequently in the differentiated type of GC than in the undifferentiated type ($p < 0.0001$) (table 1). Localized distribution of connexin 30-positive GC cells in tumors that had more than 1 histological component were also often observed in differentiated GC components rather than in undifferentiated components.

Furthermore, connexin 30 staining showed a significant inverse correlation with the depth of invasion ($p < 0.0001$), lymph node metastasis ($p = 0.0123$), and TNM stage ($p = 0.0014$). There was no significant association between connexin 30 staining and other parameters (age, gender, or M grade).

Association between Connexin 30 Expression and Gastric/Intestinal Mucin Markers

We next investigated the association between connexin 30 expression and various markers determining the

Table 2. Relationship between connexin 30 expression and gastric/intestinal mucin markers in 169 GC cases

	Connexin 30 expression		p value
	positive (n = 47)	negative (n = 122)	
MUC5AC			
Positive	17 (21)	63	NS
Negative	30 (34)	59	
MUC6			
Positive	13 (39)	20	NS
Negative	34 (25)	102	
MUC2			
Positive	33 (55)	27	<0.0001
Negative	14 (13)	95	
CD10			
Positive	14 (40)	21	NS
Negative	33 (25)	101	

Figures in parentheses are percentages. p values were calculated by Fisher's exact test. NS = Not significant.

gastric/intestinal phenotypes. Out of the 169 cases examined, each molecule was detected in 80 (47%) cases for MUC5AC, 33 (20%) cases for MUC6, 60 (36%) cases for MUC2, and 35 (21%) cases for CD10. The 169 GC cases were classified into 4 phenotypes: 50 (30%) were the gastric phenotype, 41 (24%) were the gastric and intestinal mixed phenotype, 42 (25%) were the intestinal phenotype, and 36 (21%) were the unclassified phenotype. The positive expression of connexin 30 was significantly more frequent in MUC2-positive cases than in MUC2-negative cases ($p < 0.0001$) (table 2). In immunohistochemical staining, the localized distribution of connexin 30 and MUC2 was partly contiguous (fig. 2c, d). Double immunohistochemical staining, however, did not show a coexpression of connexin 30 with MUC2 in any of the tumor cells (fig. 2e). On the other hand, there was no clear relationship between the expression of connexin 30 and other markers (MUC5AC, MUC6, and CD10) (table 2). Connexin 30 expression occurred more frequently in the intestinal phenotype (48%) than in other phenotypes (21%) of GC ($p = 0.0015$) (fig. 2g).

Discussion

Evidence of altered connexin expression in various human malignancies has been accumulating. With regard to the function of connexin in carcinogenesis, there have been several reports of inhibitory effects on the growth of cancer cells [50–53], and transfection and the forced expression of connexin 30 in glioma cell lines has been reported to induce the suppression of tumor growth in vitro [54, 55]. In the present study, we found that approximately 30% of GC cases displayed connexin 30 expression, while nonneoplastic gastric mucosa did not express connexin 30. Furthermore, there was a significant inverse association between connexin 30 expression and tumor progression. Once malignant formation is completed, connexin 30 might inhibit GC cell growth and invasion. In addition, we observed a significant inverse association between connexin 30 expression and the presence of metastasis in the regional lymph nodes. Saunders et al. [56], studying the correlation between the metastatic potential of breast cancer cells and gap junctional communication, showed that the disruption of homospecific or heterospecific GJIC contributes to metastatic potential, but mechanisms by which altered connexin expression and GJIC might contribute to this process are unclear and require future studies. Based on our results, we suggest that the aberrant expression of connexin 30 in GC might not play a role in the metastatic efficiency of malignant cells. The present study showed a higher expression of connexin 30 in the differentiated type of GC compared with the undifferentiated type. This may reflect a loss of the ability to produce this protein along with a decrease in histological differentiation in neoplastic cells. Furthermore, in some cases of GC, we observed a cytoplasmic staining pattern of connexin 30. Previous studies reported that connexin 26 translocated from the cell membrane to the cytoplasm in tumor cells [30, 33]. Furthermore, human connexin 26 and connexin 30 were reported to form functional heteromeric and heterotypic channels [57]. These findings suggest that altered expressions of connexin 30 such as a decrease in functional gap junctions and changed localization of connexin 30 are early events during the development of GC. Although the precise function of cytoplasmic connexin 30 is as yet unclear, one possibility is that the cytoplasmic accumulation of connexin 30 may be a prerequisite for the execution of its role in the cell membrane, contributing to GJIC as needed.

In the present study, the positive expression of connexin 30 showed significant correlation with the positive expression of MUC2. However, there is no previous report showing a direct association between connexin 30 and MUC2. Goblet cells in intestinal metaplasia were positive for MUC2, but scarcely expressed connexin 30. Yamamoto et al. [58] previously reported that connexin

32 might be controlled at the transcriptional level via CDX2. Therefore, connexin 30 might also be regulated by CDX2 and displayed the intestinal phenotype of GC. Further studies should be performed in the near future to elucidate a role for CDX2 in the regulation of connexin 30 in GC.

In summary, we revealed that GC with connexin 30 expression demonstrates an intestinal phenotype that is significantly MUC2-positive in expression. Connexin 30 may be a novel differentiation marker mediating the biological behavior of the intestinal phenotype of GC.

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Original Article

Immunohistochemical analysis of colorectal cancer with gastric phenotype: Claudin-18 is associated with poor prognosis

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Claudin-18 plays a key role in constructing tight junctions, and altered claudin-18 expression has been documented in various human malignancies; however, little is known about the biological significance of claudin-18 in colorectal cancer (CRC). The aim of this study is to investigate the significance of claudin-18 expression in CRC and its association with clinicopathological factors. We performed clinicopathological analysis of claudin-18 expression in a total of 569 CRCs by immunohistochemistry. Moreover, we investigated the association between claudin-18 and various markers including gastric/intestinal phenotype (MUC5AC, MUC6, MUC2 and CD10), CDX2, claudin-3, claudin-4, p53 and Ki-67.

Claudin-18 expression was detected in 21 of the 569 CRCs (4%) and was seen exclusively on the cell membrane. Positive expression of claudin-18 showed a significant correlation with positive expression of MUC5AC ($P < 0.0001$) and negative expression of CDX2 ($P = 0.0013$). The prognosis of patients with positive claudin-18 expression was significantly poorer than in negative cases ($P = 0.0106$). Multivariate analysis revealed that T grade, M grade and claudin-18 expression were independent predictors of survival in patients with CRC. We revealed that claudin-18 expression correlates with poor survival in patients with CRC and is associated with the gastric phenotype.

Key words: claudin-18, colorectal cancer, gastric phenotype, MUC5AC, prognosis

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Colorectal cancer (CRC) is one of the most common malignancies worldwide, and its incidence has increased in recent years.[1] Several molecules associated with carcinogenesis and tumor progression have been identified;[2–4] however, these mechanisms remain unclear.

Claudin proteins, a family of proteins comprising at least 24 members, are components of tight junction strands that regulate paracellular transport and lateral diffusion of membrane lipids and proteins.[5] Claudins are expressed in an organ-specific manner. Claudin-18 has two alternatively spliced variants in mice: variant 1 (claudin-18a1) is expressed in the lung, whereas variant 2 (claudin-18a2) is expressed in the stomach.[6] In normal human tissues, expression of claudin-18a2 is confined to gastric epithelial cells (foveolar, endocrine, parietal, and chief cells) and duodenal Paneth cells,[7] and not in other organs including esophagus, colon, pancreas, lung, and so on.[8] However, altered claudin-18 expression has been documented in various diseases. Expression of claudin-18 is increased in both experimental colitis and human inflammatory bowel disease.[9] Frequent ectopic activation of claudin-18 was reported in pancreatic, esophageal, ovarian, and lung tumors, using quantitative reverse transcription-PCR.[8] We previously reported that expression of claudin-18 is retained in about half of gastric cancers, which correlated with a survival benefit.[7] In addition, we showed ectopic expression of claudin-18 in signet ring cell carcinoma of CRC.[10] However, little is known about the clinicopathological significance of claudin-18 in CRC. Moreover, claudin-3 and claudin-4 were reported to express in normal colon mucosa and CRC.[11,12] Claudin-18 has been defined as gastric claudin, and claudin-3 and claudin-4 as intestinal claudin in gastric cancer, and it has been reported that classification of gastric cancers using gastric and intestinal claudins is a good biomarker for assessing the risk of poor prognosis.[13]

Mucin genes have been shown to be altered in various epithelial cancers,[14] and gastric cancers are classified as having a gastric, gastric and intestinal mixed, or intestinal phenotype depending on the expression of mucin phenotypic markers.[15–17] Aberrant expression of mucin genes has also been observed in CRC.[14,18,19] Patients with long-standing ulcerative colitis (UC) are at a high risk of development of CRC,[20] and MUC5AC and MUC6 are expressed in UC-associated CRC.[21] It has also been reported that MUC5AC expression was observed during colon carcinogenesis in rats.[22] Furthermore, MUC5AC expression has been reported to be associated with poor prognosis.[23]

The caudal homeobox 2 gene (CDX2) is a homeobox transcription factor that plays a master role in intestinal differentiation and homeostasis in the colon.[24] About 85% of CRCs express CDX2 immunohistochemically, and this is inversely associated with tumor stage.[25]

The present study represents the first detailed analysis of claudin-18 expression in CRC. To clarify the pattern of expression and localization of claudin-18 in CRC, we performed immunohistochemical analysis of surgically resected CRC samples. In addition, we investigated the association between claudin-18 and various markers including gastric/intestinal phenotype (MUC5AC, MUC6, MUC2, CD10), CDX2, other claudins (claudin-3 and claudin-4), p53 and Ki-67. Furthermore, we also evaluated the relationship between claudin-18 expression and patients' prognosis.

MATERIALS AND METHODS

Tissue samples

Primary tumor samples were collected from 569 patients with CRC (305 men and 264 women; age range, 46–93 years; mean, 68 years). Patients were treated at the Hiroshima University Hospital, Hiroshima, Japan, or affiliated hospitals. For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from 569 patients who had undergone surgical excision for CRC. The 569 CRC cases included 395 samples on a tissue microarray (TMA) using a Tissue Microarrayer (AZUMAYA KIN-1, Tokyo, Japan). The two most representative tumor areas to be sampled for the TMAs were carefully selected in each case. The 569 CRC cases were histologically classified as 308 well differentiated, 246 moderately differentiated, 11 poorly differentiated, and 4 mucinous adenocarcinomas, according to the World Health Organization (WHO) classification.[26] Tumor staging was carried out according to the TNM classification[27] and Dukes' classification. Information on patient survival was available for 97 patients with advanced CRC (Dukes' stage B to C). Because written informed consent was not obtained, 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.

Antibodies

Anti-claudin-18 antibody (C-term) was purchased from Invitrogen/Zymed Laboratories Inc. (San Francisco, CA, USA). This antibody was the same as that used in our previous study,[7] and recognizes only claudin-18a2. We used four antibodies for analysis of the CRC phenotypes: anti-MUC5AC (Novocastra, Newcastle, UK) as a marker of gastric foveolar epithelial cells, anti-MUC6 (Novocastra) as a marker of pyloric gland cells, anti-MUC2 (Novocastra) as a marker of goblet cells in the small intestine and colorectum, anti-CD10 (Novocastra) as a marker of microvilli of absorptive cells in the small intestine and colorectum. We used anti-CDX2 (BioGenex, San Ramon, CA, USA) as a marker of differentiation of intestinal epithelial cells, anti-Ki-67 (Dako, Carpinteria, CA, USA), and anti-p53 (Novocastra). Furthermore, anti-claudin-3 and anti-claudin-4 antibodies were purchased from Invitrogen/Zymed Laboratories Inc.

Immunohistochemistry

A Dako LSAB Kit (Dako) was used for immunohistochemical analysis. In brief, sections were pretreated by microwave treatment in citrate buffer for 15 min to retrieve antigenicity. After peroxidase activity was blocked with 3% H_2O_2-methanol for 10 min, sections were incubated with normal goat serum (Dako) for 20 min to block non-specific antibody binding sites. Sections were incubated with the following primary antibodies: anti-claudin-18 (diluted 1:50), anti-MUC5AC (1:50), anti-MUC6 (1:50), anti-MUC2 (1:50), anti-CD10 (1:50), anti-CDX2 (1:20), anti-claudin-3 (1:50), anti-claudin-4 (1:50), anti-Ki-67 (1:50) and anti-p53 (1:50). Sections were incubated with primary antibody for 1 h at 25°C, followed by incubations with biotinylated anti-rabbit/mouse IgG and peroxidase labelled streptavidin for 10 min each. Staining was completed with a 10-min incubation with the substrate-chromogen solution. The sections were counterstained with 0.1% haematoxylin.

Claudin-18, CDX2 and p53 staining was classified according to the percentage of stained cancer cells. Expression was considered to be 'negative' if <10% of cancer cells were stained. When at least 10% of cancer cells were stained, the result of immunostaining was considered 'positive.' Expression of claudin-3 and claudin-4 was considered to be 'reduced' if less than 50% of cancer cells were stained. When at least 50% of cancer cells were stained, the immunostain-

ing was considered 'preserved'. Ki-67 expression was classified into two groups according to the percentage of stained cancer cells.

The CRC cases were classified into four phenotypes: gastric phenotype, intestinal phenotype, gastric and intestinal mixed phenotype, and unclassified phenotype. The criteria[28] for classification of gastric phenotype and intestinal phenotype were as follows. The CRCs in which more than 10% of the cells displayed the gastric or intestinal epithelial cell phenotype were gastric phenotype or intestinal phenotype cancers, respectively. Those sections that showed both gastric and intestinal phenotypes were classified as gastric and intestinal mixed phenotype, and those that lacked both the gastric and the intestinal phenotypes were classified as unclassified phenotype.

For the TMAs, staining was also considered positive on the same basis as described above.

Double immunofluorescence staining

Double-immunofluorescence staining was performed as described previously.[29] Alexa Fluor 488-conjugated chicken anti-rabbit IgG and Alexa Fluor 546-conjugated goat anti-mouse IgG were used as secondary antibodies (Molecular Probes, Eugene, OR, USA).

Statistical methods

Correlations between clinicopathologic parameters and claudin-18 staining were analyzed by Fisher's exact test. Kaplan-Meier survival curves were constructed for claudin-18, MUC5AC, MUC6, MUC2, CD10 or CDX2-positive and -negative patients to compare survival between both groups. The differences in survival curves between groups were tested for statistical significance by the log-rank test.[30] Cox proportional hazards multivariate model was used to examine the association of clinicopathologic factors and the expression of claudin-18 with survival. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Immunohistochemical analysis of claudin-18 in CRC

We performed immunostaining of claudin-18 in CRC. Claudin-18 expression was detected in 21 of the 569 CRCs (4%) and was seen exclusively on the cell membrane (Fig. 1a). There was no difference in claudin-18 expression levels between intratumor areas and infiltrative margins, and in the presence or absence of vessel infiltration. Corresponding non-neoplastic colorectal mucosa and adenomas of the colon did not express claudin-18. Next, we analyzed the relationship between claudin-18 expression and clinicopathologic characteristics. Expression of claudin-18 was not correlated with T grade, N grade, tumor staging, or histological type (Table 1).

Association of expression between claudin-18 and various molecules including mucins, CDX2, other claudins, p53 and Ki-67

We next investigated the association between claudin-18 expression and various markers determining gastric/intestinal phenotype. Out of the 569 cases examined, each molecule was detected in 86 (15%) cases for MUC5AC, 11 (2%) cases for MUC6, 370 (65%) cases for MUC2, 200 (35%) cases for CD10. 569 CRC cases were classified into four phenotypes: 14 (2%) gastric phenotype, 76 (13%) gastric and intestinal mixed phenotype, 389 (68%) intestinal phenotype, and 90 (16%) unclassified phenotype. Positive expression of claudin-18 was significantly more frequent in MUC5AC-positive cases than MUC5AC-negative cases ($P < 0.0001$) (Table 2). In immunohistochemical staining, coexpression of claudin-18 and MUC5AC was often detected (Fig. 1a,b). Double-immunohistochemical staining also showed coexpression of claudin-18 with MUC5AC in many tumor cells (Fig. 1c). On the other hand, CDX2 was detected in 448 of the 569 (79%) cases, and positive expression of claudin-18 was significantly more frequent in CDX2-negative cases than CDX2-positive cases ($P = 0.0013$) (Table 2). There was no clear relationship between expression of claudin-18 and other markers (MUC6, MUC2, CD10, Ki-67 and p53) (Table 2). Moreover, positive expression of claudin-18 (gastric claudin) also showed no significant correlation with reduced expression of claudin-3 and claudin-4 (intestinal claudin).

Morphological characteristics of claudin-18-positive-MUC5AC-positive CRC in comparison with claudin-18-negative-MUC5AC-positive CRC

Furthermore, we analyzed morphological differences between 13 claudin and 18-positive-MUC5AC-positive CRC cases and 73 claudin-18-negative-MUC5AC-positive CRC cases. In the former, 6 of the 13 (46%) cases showed cohesive and microglandular structures with scant cytoplasm and nuclear atypia (Fig. 1d,e). They resembled gastric tubular adenocarcinoma in appearance, the components of which also showed gastric phenotype immunohistochemically. In the latter, only 5 of the 73 (7%) cases showed such struc-

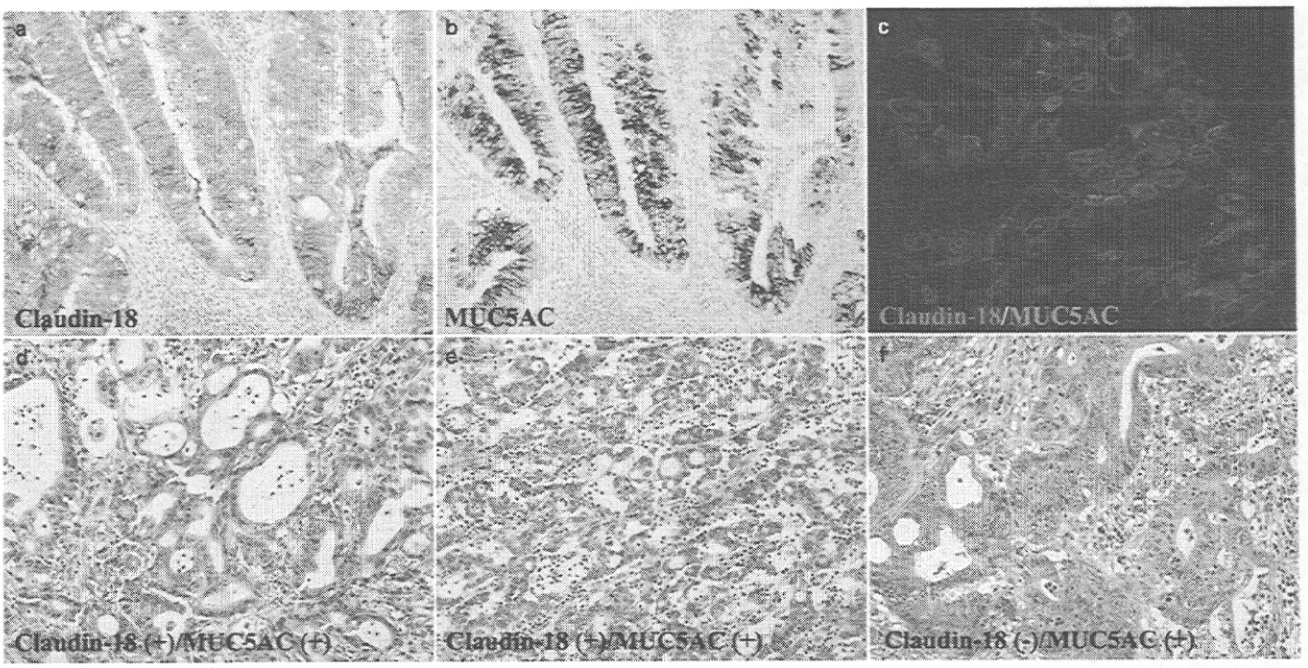

Figure 1 Immunohistochemical staining of claudin-18 and MUC5AC in colorectal cancer (CRC) tissues. (**a**) Immunohistochemistry of claudin-18. Claudin-18 was detected in cell membranes. (**b**) Immunohistochemistry of MUC5AC. MUC5AC was detected in the cytoplasm. (**c**) Double-immunostaining of claudin-18 (red) and MUC5AC (green). Nuclei are stained with 4; 6-diamidino-2-phenylindole (DAPI; blue). (**d,e**) Some of claudin-18-positive-MUC5AC-positive CRC showed cohesive and microglandular structures with scant cytoplasm and nuclear atypia. The resemblance to gastric tubular adenocarcinoma is remarkable. (**f**) Most of claudin-18-negative-MUC5AC-positive CRC revealed irregular formation of glands with relatively abundant and eosinophilic cytoplasm, prominent nuclei and loss of polarity. Original magnification: (**a,b,d–f**) × 200; (**c**) × 800.

tures, and remaining cases revealed common form of conventional CRC which have irregular formation of glands with relatively abundant and eosinophilic cytoplasm, prominent nuclei and loss of polarity (Fig. 1f).

Relationship between expression of claudin-18, mucins and CDX2 in CRC and patients' prognosis

We also examined the relationship between survival and expression of claudin-18, mucins and CDX2 in advanced CRC (Dukes' stage B to C, $n = 97$). The prognosis of patients with positive claudin-18 or MUC5AC expression was significantly poorer than in the negative cases ($P = 0.0106$ or $P = 0.0462$, respectively, log-rank test) (Fig. 2a,b). In contrast, the prognosis of patients with negative CDX2 expression was significantly poorer than in the positive cases ($P = 0.0044$, log-rank test) (Fig. 2c). The percentage of claudin-18 positive area within CRC between >50% and 10–50% showed no significant difference in prognosis. In the meantime, the prognosis of patients with positive MUC2 or CD10 expression was not significantly poorer than in the negative cases. Next, the Cox proportional hazards multivariate model was used to examine the association of clinicopathologic factors and the expression of claudin-18 with survival. Multivariate analysis revealed that T grade, M grade and claudin-18 expression were independent predictors of survival in patients with CRC (Table 3).

DISCUSSION

Evidence of altered claudin expression in various human malignancies has been accumulating. In ovarian cancer, claudin-3 and claudin-4 proteins are highly overexpressed[31] and overexpression of these claudins increases cell invasion and motality,[32] whereas reduced or loss of expression of claudin family members has been found to promote cell invasion and metastasis in malignant tumors, including those of the breast,[33] pancreas[34] and gastrointestinal tract.[12] Ectopic expression of claudin-18 in CRC has also been reported recently.[8,10] However, little is known about the biological significance of claudin-18 in CRC. We previously reported that retained claudin-18 was correlated with a survival benefit in gastric cancer.[7] In the present study, however, ectopic expression of claudin-18 in CRC was correlated with poor survival. Seemingly, this result contradicts the previous analysis in gastric cancer. Claudin family members are crucial components of tight junction, and exhibit highly tissue specific patterns.[35] In gastric cancer, down-regulation of