This fact suggests that VEGF-C may directly influence tumor cell growth or motility via an autocrine mechanism. Therefore, we conducted the present study to clarify whether gastric cancer cells express functional VEGFR-3 and to evaluate the biological significance of VEGFR-3 expression in gastric cancer progression.

In the present study, we found that VEGFR-3 is expressed by tumor cells as well as lymphatic endothelial cells in gastric carcinoma tissues. Approximately half of our gastric cancers (17 of 36) contained tumor cells that expressed VEGFR-3 protein. On the basis of the lack of VEGF-C and VEGFR-3 expression in normal gastric epithelial cells, we concluded that *de novo* expression of VEGF-C and VEGFR-3 seems to be associated with the process of malignant transformation. Treatment of cultured KKLS cells with VEGF-C induced tyrosine phosphorylation of VEGFR-3 and then increased proliferation. It also induced expression of cyclin D1, PlGF, AMF, and AMFR. PlGF and AMF are growth factors known to regulate angiogenesis and tumor cell motility, respectively. We previously reported that VEGF-C expression is higher at the site of deepest

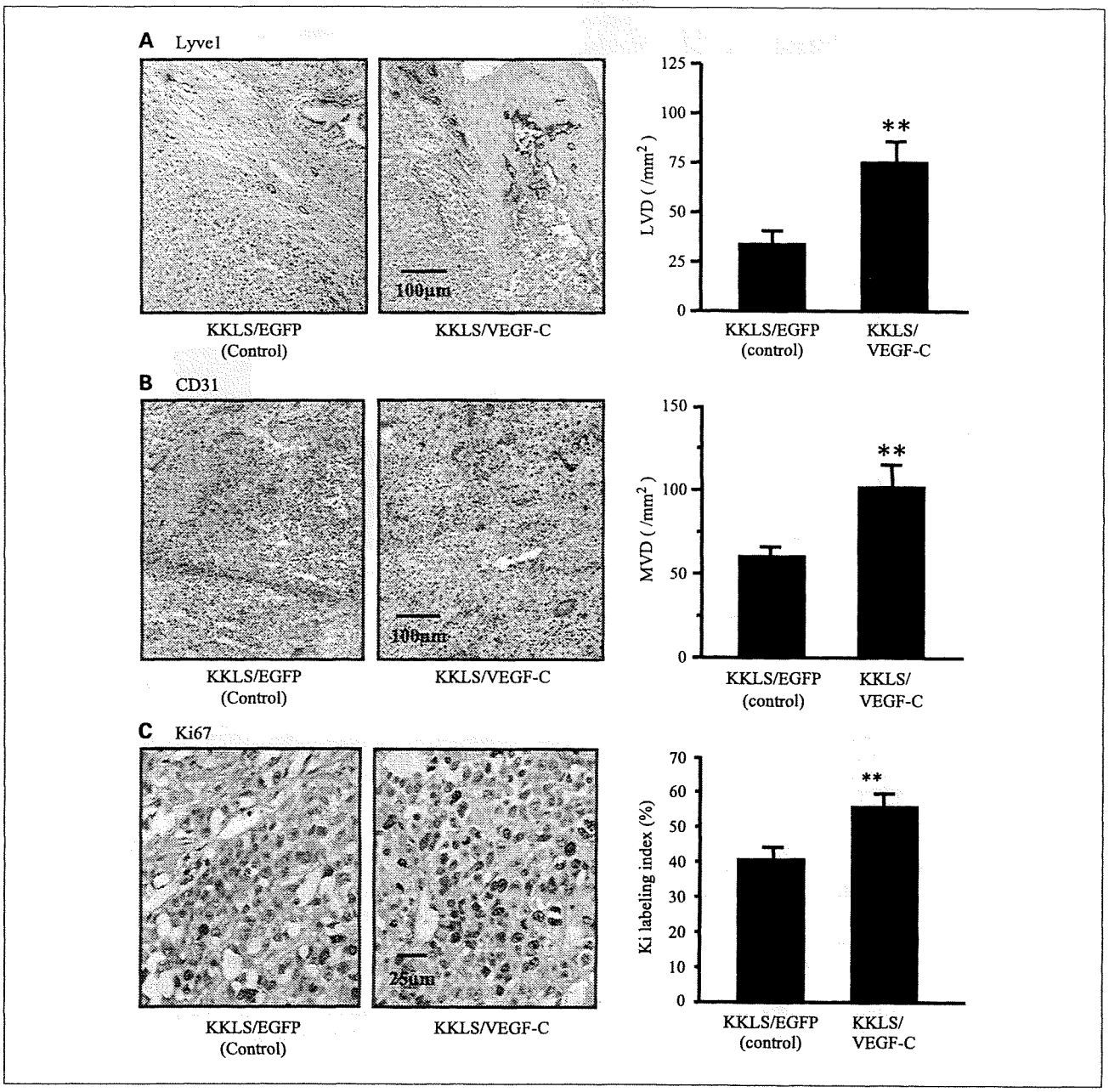

Fig. 6. Immunohistochemistry for Lyve1 (*A*), CD31 (*B*), and Ki-67 (*C*) in KKLS cells growing in the stomach of nude mice. VEGF-C-transfected KKLS tumors showed increased density of Lyve-1 – and CD31-positive vessels and higher numbers of Ki-67 – positive cells than control tumors. Right panels, quantification of Lyve-1 – positive vessels, CD31-positive vessels, and Ki-67 – positive cells in these tumors. ** $P < 0.01$; bars, SE.

penetration of the invasive tumor than in the superficial portions (16). In the present study, heterogeneous intratumoral staining was observed for VEGFR-3 and VEGF-C, with the highest levels of expression at the invasive edges. Expression of PlGF and AMF/AMFR induced by VEGF-C/VEGFR-3 signaling may play a role in progression of gastric cancer cells to an aggressive phenotype.

Makinen et al. proved the critical role of VEGF-C/VEGFR-3 signaling in the growth and survival of lymphatic endothelial cells (43). They found VEGFR-3 induces a protein kinase C–dependent p42/p44 mitogen-activated protein kinase activation and wortmannin-sensitive phosphorylation of Akt. However, the biochemical signaling pathways activated via VEGFR-3 are unknown in tumor cells. In the present study, we found treatment with VEGF-C resulted in phosphorylation of Akt but not mitogen-activated protein kinase in KKLS cells. Further studies using Akt inhibitor will be needed to clarify whether Akt indeed plays a role as a signaling molecule of the VEGF-C/VEGFR-3 axis.

To stimulate VEGF-C/VEGFR-3 signaling in an autocrine manner, we transfected a VEGF-C expression vector into KKLS cells, established stable transfectants, and transplanted VEGF-C-transfected cells and control cells into the gastric walls of nude mice (orthotopic site). Lyve1-positive vessel-like structures were found at a much higher density in KKLS/VEGF-C tumors than in control tumors. These findings are consistent with the published direct evidence for the role of VEGF-C in tumor lymphangiogenesis (28, 29). However, VEGF-C secreted by the tumor did not promote lymphatic metastasis in our present experiments. Lymph node metastasis was not observed in the KKLS/VEGF-C or the control mice. For lymphatic metastasis, tumor cells must complete multiple steps, which include lymphangiogenesis, motility, invasion, survival in the circulation, adhesion, extravasation, and proliferation (2, 3). He et al. (42) reported that tumors of lung carcinoma cells overexpressing VEGF-C contain more lymphatic vessels than vector-transfected tumors but do not have increased metastatic ability. Therefore, lymphangiogenesis induced by VEGF-C may not be the only metastasis rate-limiting factor. To survive in the lymph circulation and colonize lymph nodes, another growth factor, such as VEGF-A (44) or platelet-derived growth factor-BB (45), may be needed.

In addition to lymphangiogenesis, we observed greatly accelerated angiogenesis and *in vivo* growth of KKLS/VEGF-C cells compared with that of control cells in the present study. VEGF-C and VEGF-D can exert angiogenic activity through VEGFR-2 (46), and previous data clearly link VEGFR-2 to angiogenesis and progression of gastric cancer (47). On the other hand, Mandriota et al. reported that VEGF-C induces lymphangiogenesis, but not angiogenesis in double-transgenic mice (48). Although there is no explanation for the discrepancy, this might be due to differences in VEGF-C proteolytic processing in the different models. The stepwise proteolytic processing of VEGF-C generated several VEGF-C forms with increased activity towards VEGFR-3, but only the fully processed VEGF-C could activate VEGFR-2 (49). VEGFR-3 has been detected in both blood vessels and lymphatic vessels in tumor tissues (27, 50, 51). There are several studies that showed that antibody interference with VEGFR-3 function can inhibit tumor growth by inhibiting neoangiogenesis in various human tumor xenografts in immunocompromised mice (34, 52). We found in the present study that VEGF-C induces the expression of PlGF by KKLS cells. Expression of PlGF by KKLS cells may also play a role in tumor angiogenesis.

Beside roles in lymphangiogenesis and angiogenesis, we identified an additional role of VEGF-C/VEGFR-3 in tumor growth. Treatment with VEGF-C increased expression of cyclin D1 (a cell cycle regulator) and stimulated growth of KKLS cells *in vitro*, and we found a higher number of Ki-67–positive cells in KKLS/VEGF-C tumors than in vector control tumors. He et al. (53) reported that treatment of ectopic xenografts of lung carcinoma cells with VEGFR-3-immunoglobulin inhibits tumor growth without a reduction in microvessel density. These findings support the existence of autocrine stimulation of tumor growth by the VEGF-C/VEGFR-3 axis.

In summary, our results show that VEGF-C is an important growth factor, in addition to acting as a lymphangiogenic or angiogenic factor. Thus, we propose that interruption of the VEGF-C/VEGFR-3 axis may be a therapeutic approach for controlling disease progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Gene expression profiling with microarray and SAGE identifies PLUNC as a marker for hepatoid adenocarcinoma of the stomach

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Gastric cancer is one of the most common malignancies worldwide. In this study, we screened for genes upregulated in gastric cancer by comparing gene expression profiles from serial analysis of gene expression and microarray and identified the palate, lung, and nasal epithelium carcinoma-associated protein (*PLUNC*) gene. Immunostaining for PLUNC in 140 gastric cancer cases revealed strong and extensive staining of PLUNC in hepatoid adenocarcinoma of the stomach, whereas 7% of conventional gastric cancer cases showed focal immunostaining of PLUNC. Gastric hepatoid adenocarcinoma is an extrahepatic tumor characterized by morphologic similarities to hepatocellular carcinoma. To investigate the utility of PLUNC immunostaining in the diagnosis of gastric hepatoid adenocarcinoma, six cases of gastric hepatoid adenocarcinoma (six primary tumors and two associated liver metastases) were studied further. PLUNC staining was observed in all six primary hepatoid adenocarcinomas. PLUNC staining was observed in both the hepatoid adenocarcinoma and tubular/papillary adenocarcinoma components of primary tumors, although PLUNC staining was preferentially localized in tubular/papillary adenocarcinoma components. Staining of PLUNC was also detected in both liver metastases. PLUNC staining was not observed in 52 cases of primary hepatocellular carcinoma or in normal adult or fetal liver. These results indicate that PLUNC is a novel marker that distinguishes gastric hepatoid adenocarcinoma from primary hepatocellular carcinoma.
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Keywords: hepatoid adenocarcinoma; hepatocellular carcinoma; PLUNC; microarray; SAGE; gastric cancer

According to the World Health Organization, gastric cancer is the fourth most common malignancy worldwide, with approximately 870 000 new cases occurring every year. Mortality due to gastric cancer is second only to that due to lung cancer.[1] Cancer develops as a result of multiple genetic and epigenetic alterations.[2–4] 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 the major goals in this field.[5] To identify potential molecular markers for cancer and to better understand the development of cancer at the molecular level, comprehensive gene expression analysis may be useful. Among the comprehensive methods used to analyze transcript expression levels, array-based hybridization[6] and serial analysis of gene expression (SAGE)[7] are currently the most common approaches.

We previously performed SAGE of four primary gastric cancers.[8] From the SAGE data, we identified several gastric cancer-associated genes;[9] however, these alterations cannot completely explain the pathogenesis of gastric cancer. In addition, although gene expression profiles from SAGE and microarray have better correlations for genes with high-fold changes, the gene expression profiles from these methods show relatively poor correlations among genes with low-fold changes, suggesting that SAGE data may not yield a comprehensive gene expression profile.[10] In our previous study, the invasion/metastasis-associated genes identified by SAGE

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were quite different from those identified by microarray.[11] Therefore, we performed gene expression profiling using Affymetrix GeneChip Human Genome U133Plus 2.0 arrays of one gastric cancer sample previously analyzed by SAGE and identified several candidate gastric cancer-associated genes. Among these candidate genes, the palate, lung, and nasal epithelium carcinoma-associated protein (*PLUNC*, also known as lung-specific X protein, *LUNX*) gene is upregulated in human gastric cancer. However, little is known about the relation of PLUNC to human gastric cancer.

PLUNC was originally identified in the nasal epithelium of mouse embryo and the trachea and bronchi of adult mouse lung.[12] The human *PLUNC* gene shows a similar expression pattern, including localization to the tracheal epithelium.[13] *PLUNC* was also identified as a marker of non-small-cell lung carcinoma.[14] Immunohistochemical analysis of PLUNC in lung cancer revealed that PLUNC is commonly expressed in adenocarcinoma, mucoepidermoid carcinoma, and bronchoalveolar carcinoma and is absent from small cell carcinoma and squamous cell carcinoma.[15] Although PLUNC is a major secreted protein product in the upper respiratory tract,[15] the biologic function of PLUNC is poorly understood.

In this study, we examined the expression and distribution of PLUNC in human gastric cancer by immunohistochemistry. The relation between staining for PLUNC and clinicopathologic characteristics was examined. In addition, because we observed frequent immunostaining of PLUNC in hepatoid adenocarcinoma of the stomach, we also performed PLUNC immunostaining of primary hepatocellular carcinoma to investigate the potential utility of PLUNC immunostaining in the diagnosis of gastric hepatoid adenocarcinoma.

Materials and methods

Tissue Samples

In total, specimens from 144 cases of primary gastric cancer and 52 cases of primary hepatocellular carcinoma were collected. Patients were treated at the Hiroshima University Hospital. The histologic classification was based on the World Health Organization system. Tumor staging was according to the TNM classification system.[16] Because written informed consent was not obtained, for strict privacy protection, identifying information for all samples was removed before analysis. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government. For microarray analysis, one primary gastric cancer sample (Case P208T, 60-year-old man, T4N3M0, stage IV, poorly differentiated adenocarcinoma) and corresponding non-neoplastic mucosa were used. This gastric cancer sample was analyzed previously by SAGE for comprehensive gene expression profiling.[8] For quantitative reverse transcription (RT)-polymerase chain reaction (PCR) analysis, four gastric cancer samples and corresponding non-neoplastic mucosa samples were used. The samples were obtained during surgery at the 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 use. Samples of normal brain, spinal cord, heart, skeletal muscle, lung, stomach, small intestine, colon, liver, pancreas, kidney, bone marrow, spleen, peripheral leukocytes, and trachea were purchased from Clontech (Palo Alto, CA, USA). For western blot analysis, lysates from normal adult stomach and trachea were purchased from Clontech. For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from 144 patients who had undergone surgical excision of gastric cancer (65 women and 79 men; age range, 38–90 years; mean, 68 years). Thirty-nine of the 144 patients had early gastric cancer, and 105 had advanced gastric cancer. Early gastric cancer is limited to the mucosa or the mucosa and submucosa, regardless of nodal status. Advanced gastric cancer is a tumor that has invaded beyond the submucosa.[17] Primary gastric cancers exhibiting a hepatoid component were regarded as hepatoid adenocarcinomas. Out of 144 primary gastric cancers, 6 cases were histologically classified as hepatoid adenocarcinoma. Out of six hepatoid adenocarcinoma cases, liver metastasis samples were available for two cases. Two patients with liver metastasis were serologically negative for HBV and HCV infection and they did not show any clinical or echographic signs of cirrhosis. Both patients had no history of alcohol abuse. In addition, we used archival formalin-fixed, paraffin-embedded tissues from 52 patients who had undergone surgical excision of hepatocellular carcinoma (8 women and 44 men; age range, 44–82 years; mean, 64 years; stage I, 31 cases; stage II, 16 cases; stage III, 5 cases). Normal adult ($n=2$, 45 and 57 years) and fetal ($n=2$, 10 and 18 gestational weeks) livers were obtained at autopsy.

Microarray Analysis

One primary gastric cancer sample (P208T) and corresponding non-neoplastic mucosa were analyzed by genome-wide microarray, as described previously.[18] Here, we used Affymetrix GeneChip Human Genome U133Plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA). Each transcript on this array is represented by a set of 11 probe pairs, called the probe set. The array contains >54 000 probe sets, representing 47 400 transcripts, including 38 500 genes. Five micrograms of total RNA was used to prepare antisense biotinylated RNA with One-cycle Target Labeling and Control Reagent (Affymetrix) as per the manufacturer's instructions. In brief, first-stranded cDNA was synthesized with a T7-RNA polymerase

promoter-attached oligo(dT) primer followed by second-stranded cDNA synthesis. This cDNA was purified and served as a template in the subsequent *in vitro* T7-transcription (IVT). The IVT reaction was carried out in the presence of T7 RNA polymerase and biotinylated UTP for cRNA production. The biotinylated cRNAs were then cleaned up and fragmented. The fragmented, biotinylated cRNA was hybridized to the array (45°C for 16 h). The procedures for staining, washing, and scanning of arrays were carried out as per the instructions in the Affymetrix technical manual. The expression value (average difference, AD) of each probe was calculated with GeneChip Operating Software Version 1.1 (Affymetrix). The mean of AD values in each experiment was 1000 to reliably compare variable multiple arrays.

Quantitative RT-PCR

Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and 1 μg of total RNA was converted to cDNA with a First Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ, USA). Quantitation of *PLUNC* mRNA levels in human tissue samples was done by real-time fluorescence detection, as described previously.[19] *PLUNC* primer sequences were 5′-CAG TTGCCT TCT CTC CGA GG-3′ and 5′-CAT GGG ATG TTA CAC ACGCC-3′. PCR was performed with an SYBR Green PCR Core Reagents Kit (Applied Biosystems, Foster City, CA, 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.[20] *ACTB*-specific PCR products were amplified from the same RNA samples and served as internal controls.

Western Blotting

Western blotting was performed as described previously.[21] Lysates (40 μg) were solubilized in Laemmli sample buffer by boiling and then subjected to 12% SDS-polyacrylamide gel electrophoresis followed by electrotransfer onto a nitrocellulose filter. The filter was incubated with the primary antibody against PLUNC (goat polyclonal, dilution 1:500; R&D Systems, Abingdon, UK). Peroxidase-conjugated anti-goat IgG was used in the secondary reaction. Immunocomplexes were visualized with an ECL Western Blot Detection System (Amersham Biosciences). β-Actin (Sigma, St Louis, MO, USA) was also stained as a loading control.

Immunohistochemistry

Formalin-fixed, paraffin-embedded samples were sectioned, deparaffinized, and stained with H&E to ensure that the sectioned block contained tumor cells. Adjacent sections were then stained immunohistochemically. Antigen retrieval was done by microwave heating in citrate buffer (pH 6.0) for 30 min for PLUNC, HepPar1, cytokeratin 19 (CK19), cytokeratin 20 (CK20), and polyclonal carcinoembryonic antigen (p-CEA). After peroxidase activity was blocked with 3% H_2O_2–methanol for 10 min, sections were incubated with normal goat serum (Dako Cytomation, Carpinteria, CA, USA) for 20 min to block nonspecific antibody-binding sites. Sections were incubated with the primary antibodies against PLUNC (the same antibody used in western blotting to PLUNC, dilution 1:50), alpha-fetoprotein (AFP) (C3, 1:20; Novocastra, Newcastle, UK), HepPar1 (OCH1E5, 1:20; Dako Cytomation), CK19 (RCK108, 1:50; Dako Cytomation), CK20 (Ks20.8, 1:50; Dako Cytomation), and p-CEA (1:1000; Dako Cytomation) for 1 h at room temperature, followed by incubations with biotinylated anti-goat, anti-rabbit, or anti-mouse IgG and peroxidase-labeled streptavidin for 10 min each. Staining was completed with 10 min incubation with the substrate–chromogen solution. The sections were counterstained with 0.1% hematoxylin. The staining results were recorded in semiquantitative fashion as follows: 0, absence of staining; 1+, any tumor cell stained to 10% of tumor cells stained; 2+, 11–50% of tumor cells stained; and 3+, more than 50% of the tumor cells stained.

Statistical Methods

Associations between clinicopathologic parameters and PLUNC expression were analyzed by χ^2-test. Kaplan–Meier survival curves were constructed for PLUNC-positive and PLUNC-negative patients. Survival rates were compared between PLUNC-positive and PLUNC-negative groups. Differences between survival curves were tested for statistical significance by log-rank test.[22] A *P*-value of less than 0.05 was considered statistically significant.

Results

Comparison of Gene Expression Profiles from Microarray and SAGE

The gene expression profiles obtained from the gastric cancer sample (P208T) and corresponding non-neoplastic gastric mucosa sample were compared. To identify ideal biomarkers for gastric cancer, we focused on genes that showed significantly increased expression in gastric cancer. The top 20 genes that showed higher expression in the gastric cancer sample than in the corresponding non-neoplastic gastric mucosa sample by microarray analysis are listed in Table 1. The gene showing the greatest increase in expression in the gastric cancer sample by microarray was *PLUNC*. Increased expression of the *MAGE* genes

Table 1 Comparison of gene expression profiles from microarray and SAGE

Microarray				*SAGE*[a]		
Symbol	*Intensity*		*Fold*	*Symbol*	*Tag counts*	
	P208T[b]	*Non-neoplastic mucosa*			*P208T*[b]	*Normal stomach*[c]
PLUNC	74421	31	2401	*TSG101*	41	0
MAGEA12	7492	8	937	Transcribed locus	32	0
LACRT	15873	21	756	*CTNND1, CYP20A1*	28	0
MAGEA6	15966	31	515	*BCL2L2*	50	1
MAGEA2	4266	12	356	No match	24	0
MAGEA3	18963	59	321	*TUSC3*	21	0
FN1	2818	9	313	*ELOVL5*	20	0
NTRK2	2123	7	303	*S100A9*	17	0
HOXA10	4481	16	280	*KRTHB1, PHYHD1*	16	0
COL11A1	2303	10	230	*PAWR*	16	0
FGFR2	5593	25	224	*USP7*	14	0
C20orf186	4711	21	224	*KRT7, SH3BP2*	14	0
NXF3	1820	10	182	*CTSL*	14	0
LECT1	3348	24	140	*SEC11L1, WIF1*	26	1
SPP1	288	2	144	No match	12	0
SH3BP4	1214	10	121	*TFF3*	12	0
HM74	22034	209	105	*TKT*	12	0
FLJ20300	9617	96	100	*DNAJC10*	12	0
LOC284527	676	7	97	No match	11	0
SLC19A3	1631	17	96	*DDOST*	11	0

[a]The 20 most upregulated genes in gastric cancer (P208T) compared with normal stomach by SAGE analysis was determined previously.[8]
[b]Gastric cancer sample (60-year-old man, T4N3M0, stage IV, poorly differentiated adenocarcinoma).
[c]SAGE data from normal gastric epithelia (GSM784, SAGE normal gastric body epithelial, El-Rifai *et al*[23]).

(*MAGEA2*, *MAGEA3*, *MAGEA6*, and *MAGEA12*),[24] *FN1* (encoding fibronectin 1),[25] and *FGFR2* (encoding fibroblast growth factor 2)[26] has been reported previously. The 20 genes with the greatest increase in expression in gastric cancer compared with normal stomach[23] by SAGE analysis are shown in Table 1. Surprisingly, the 20 most upregulated genes identified by microarray were quite different from those identified by SAGE, indicating that genes upregulated in gastric cancer are not always detected by SAGE. We reviewed the expression level of *PLUNC* with our SAGE data. The sequence of the SAGE tag that represents *PLUNC* was TGCCTCACCT, and this sequence appeared only three times in the P208T SAGE data. Because expression of *PLUNC* has not been investigated in gastric cancer, we decided to analyze *PLUNC* expression in gastric cancer. Quantitative RT-PCR was performed to investigate the specificity of *PLUNC* expression. As shown in Figure 1a, *PLUNC* expression was clearly detected in adult trachea and to a lesser extent in adult lung. Expression of *PLUNC* was not detected in any other normal organs, including stomach. These results are consistent with those of a previous report.[13] *PLUNC* was expressed in P208T, whereas other gastric cancer tissue samples did not express *PLUNC*.

Immunohistochemical Analysis of PLUNC in Gastric Cancer

We observed upregulation of *PLUNC* mRNA in gastric cancer tissue; however, the expression pattern of PLUNC protein in gastric cancer remains unclear. To address this issue, we performed immunostaining of PLUNC. We first tested the specificity of the anti-PLUNC antibody. Western blotting of lysates from normal adult trachea and normal stomach was performed. The anti-PLUNC antibody detected an approximately 27 kDa band in adult trachea (Figure 1b). These results are consistent with our quantitative RT-PCR data for *PLUNC*. Immunostaining of P208T revealed that PLUNC was present in cytoplasm of tumor cells but not in corresponding non-neoplastic mucosa (Figure 1c). Stromal cells showed weak or no staining of PLUNC. Staining of PLUNC was not observed in the remaining three gastric cancer samples used for quantitative RT-PCR. Taken together, these data show that this anti-PLUNC antibody specifically recognizes PLUNC protein.

We next performed immunohistochemical analysis of PLUNC in 140 human gastric cancer tissue samples. Strong cytoplasmic staining of PLUNC was found in 12 (9%) of 140 gastric cancer samples. Only tumor cells were positive for PLUNC. Stromal cells were not stained. Corresponding non-neoplastic gastric mucosa, including intestinal metaplasia, was not stained. In gastric cancer tissue, staining of PLUNC was heterogeneous, and among 12 PLUNC-positive gastric cancer cases, less than 1% of tumor cells were stained in 9 gastric cancer cases. In the remaining three gastric cancer cases, more than 30% of tumor cells were stained. We then analyzed the relation of PLUNC expression to

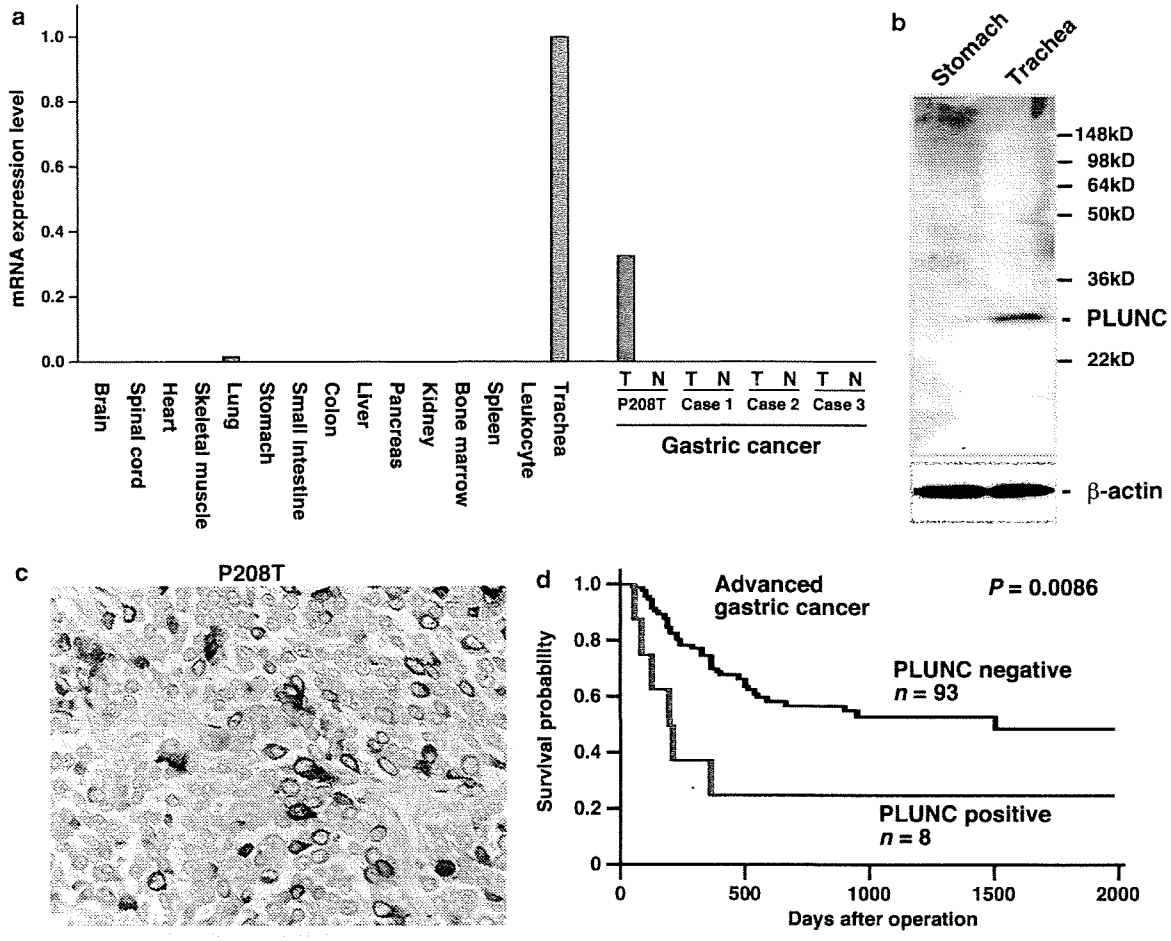

Figure 1 (**a**) Quantitative RT-PCR analysis of *PLUNC* in 15 normal tissues, four gastric cancer cases (T), and corresponding non-neoplastic mucosa (N). Case P208T was analyzed by SAGE and microarray. The units are arbitrary, and we calculated the level of *PLUNC* mRNA expression by standardization to 1.0 μg of total RNA from trachea as 1.0. (**b**) Western blot analysis of PLUNC with anti-PLUNC antibody. An approximately 27 kDa band is present in lysate of normal trachea. (**c**) Immunostaining of PLUNC in gastric cancer (Case P208T). Focal-positive staining for PLUNC in the cytoplasm is observed. (**d**) Prognostic value of PLUNC staining. Patients with gastric cancer showing PLUNC expression had a significantly worse survival rate than patients without PLUNC expression ($P = 0.0086$, log-rank test).

clinicopathologic characteristics. No correlation was found between PLUNC expression and depth of invasion, lymph node metastasis, or tumor stage (Table 2). In contrast, among 101 advanced gastric cancer cases followed up at the hospital, patients with PLUNC-positive gastric cancer had a significantly worse survival rate than those patients with PLUNC-negative gastric cancer ($P = 0.0086$, log-rank test, Figure 1d). Interestingly, of the three gastric cancer cases with more than 30% of tumor cells stained, two cases showed hepatoid adenocarcinoma of the stomach. The remaining one case was P208T and we confirmed that P208T was not gastric hepatoid adenocarcinoma. It has been reported that gastric hepatoid adenocarcinoma shows canalicular staining pattern of p-CEA,[27] and we confirmed that P208T displayed cytoplasmic pattern of p-CEA (data not shown). PLUNC staining was found more frequently in gastric hepatoid adenocarcinoma (2/2, 100%) than in other types (well and poorly differentiated adenocarcinoma) of gastric cancer (10/138, 7%, $P = 0.0007$, χ^2-test).

Immunohistochemical Analysis of PLUNC in Hepatoid Adenocarcinoma of the Stomach

It is well known that gastric hepatoid adenocarcinoma has an aggressive clinical course and poor survival.[28,29] Its biologic behavior is mainly due to its extensive hematogenous metastasis to the liver and early and frequent involvement of the lymph nodes. Gastric hepatoid adenocarcinoma closely mimics and is even indistinguishable from hepatocellular carcinoma. This makes differential diagnosis challenging, especially when the primary tumor is unknown and the first diagnosis has to be established by liver biopsy. Therefore, there is an urgent need for specific biomarkers of hepatoid adenocarcinoma. We performed immunohistochemical analysis

Table 2 Association of PLUNC expression with clinicopathologic features of gastric cancer

	PLUNC expression (%)		P-*value*[a]
	Positive	*Negative*	
T grade			
T1	4 (10)	35	0.9157
T2/T3/T4	8 (8)	93	
N grade			
N0	5 (7)	62	0.8833
N1/N2/N3	7 (10)	66	
M grade			
M0	11 (8)	120	0.7784
M1	1 (11)	8	
Stage			
Stage I	4 (7)	56	0.6394
Stage II	3 (12)	22	
Stage III	2 (8)	23	
Stage IV	3 (10)	27	
Histologic type			
Well-differentiated adenocarcinoma	6 (7)	75	0.0007[b]
Poorly differentiated adenocarcinoma	4 (7)	53	
Hepatoid adenocarcinoma	2 (100)	0	

[a]χ^2-Test.
[b]Well-differentiated and poorly differentiated adenocarcinoma *vs* hepatoid adenocarcinoma.

of PLUNC in four additional cases of hepatoid adenocarcinoma to investigate the potential utility of PLUNC immunostaining in the diagnosis of hepatoid adenocarcinoma. Among six cases of hepatoid adenocarcinoma (two cases from immunohistochemical analysis in 140 gastric cancer cases plus four additional cases), staining of PLUNC was observed in all six primary tumors. It has been reported that most hepatoid adenocarcinoma cases contain coexistent tubular or papillary adenocarcinoma components within the tumor.[30] All six gastric hepatoid adenocarcinoma cases contained both tubular/papillary adenocarcinoma and hepatoid adenocarcinoma components. out of the six hepatoid adenocarcinoma cases, PLUNC staining was observed in both the hepatoid adenocarcinoma component (Figure 2a and b) and the tubular/papillary adenocarcinoma component (Figure 2c and d). We confirmed that hepatoid adenocarcinoma component showed a canalicular pattern of p-CEA staining (Figure 2a, inset). The PLUNC staining was preferentially found in the tubular/papillary adenocarcinoma component of the primary tumors. In some cases, even when PLUNC-positive tumor cells were found in the tubular/papillary adenocarcinoma component near the hepatoid adenocarcinoma component, PLUNC was not stained in the hepatoid adenocarcinoma component (Figure 2e).Out of the six cases of hepatoid adenocarcinoma, the hepatoid adenocarcinoma component contained 5–10% PLUNC-positive tumor cells, whereas tubular/papillary adenocarcinoma component contained $\leq$70% PLUNC-positive tumor cells (Table 3). Out of the six cases of hepatoid adenocarcinoma, liver metastases from two cases were available for immunohistochemistry and were composed of hepatoid adenocarcinoma component. PLUNC staining was observed in both liver metastases (Figure 2f). One case showed 5% PLUNC-positive tumor cells, and another case contained 20% PLUNC-positive tumor cells within the metastatic tumor. Adjacent non-neoplastic liver tissues were not stained for PLUNC (Figure 2f). We also performed immunohistochemical analysis of PLUNC in 52 cases of hepatocellular carcinoma, 2 cases of normal adult liver, and 2 cases of fetal liver; however, staining of PLUNC was not observed (data not shown). These results indicate that PLUNC is a good marker to distinguish gastric hepatoid adenocarcinoma from primary hepatocellular carcinoma.

Immunohistochemical Analysis of AFP, HepPar1, CK19, and CK20 in Hepatoid Adenocarcinoma of the Stomach

Because production of AFP is usually observed in gastric hepatoid adenocarcinoma, we performed immunostaining of AFP. The results are summarized in Table 3. Although all six hepatoid adenocarcinoma cases were positive for AFP, AFP-positive tumor cells were not positive for PLUNC. AFP-positive tumor cells were observed in hepatoid adenocarcinoma components, whereas PLUNC-positive tumor cells were found mainly in tubular/papillary adenocarcinoma components (Figure 3a and b). Even when PLUNC-positive tumor cells were found in hepatoid adenocarcinoma components, PLUNC-positive tumor cells did not express AFP (Figure 3c and d).

It was previously reported that immunohistochemical analyses of HepPar1, CK19, or CK20 are useful to distinguish gastric hepatoid adenocarcinoma from primary hepatocellular carcinoma.[27] HepPar1 staining is detected more frequently in hepatocellular carcinoma than in gastric hepatoid adenocarcinoma.[27] Staining for CK19 and CK20 is detected more frequently in gastric hepatoid adenocarcinoma than in hepatocellular carcinoma.[27] Therefore, we also performed immunostaining of HepPar1, CK19, and CK20 in gastric hepatoid adenocarcinoma and hepatocellular carcinoma (Table 3). The overall results are summarized in Table 4. Out of six cases of gastric hepatoid adenocarcinoma, four cases showed focal positivity for HepPar1, and all four hepatoid adenocarcinoma cases showed less than 10% HepPar1-positive tumor cells. The remaining two hepatoid adenocarcinoma cases did not express HepPar1. Staining of HepPar1 was also detected in both liver metastases of hepatoid

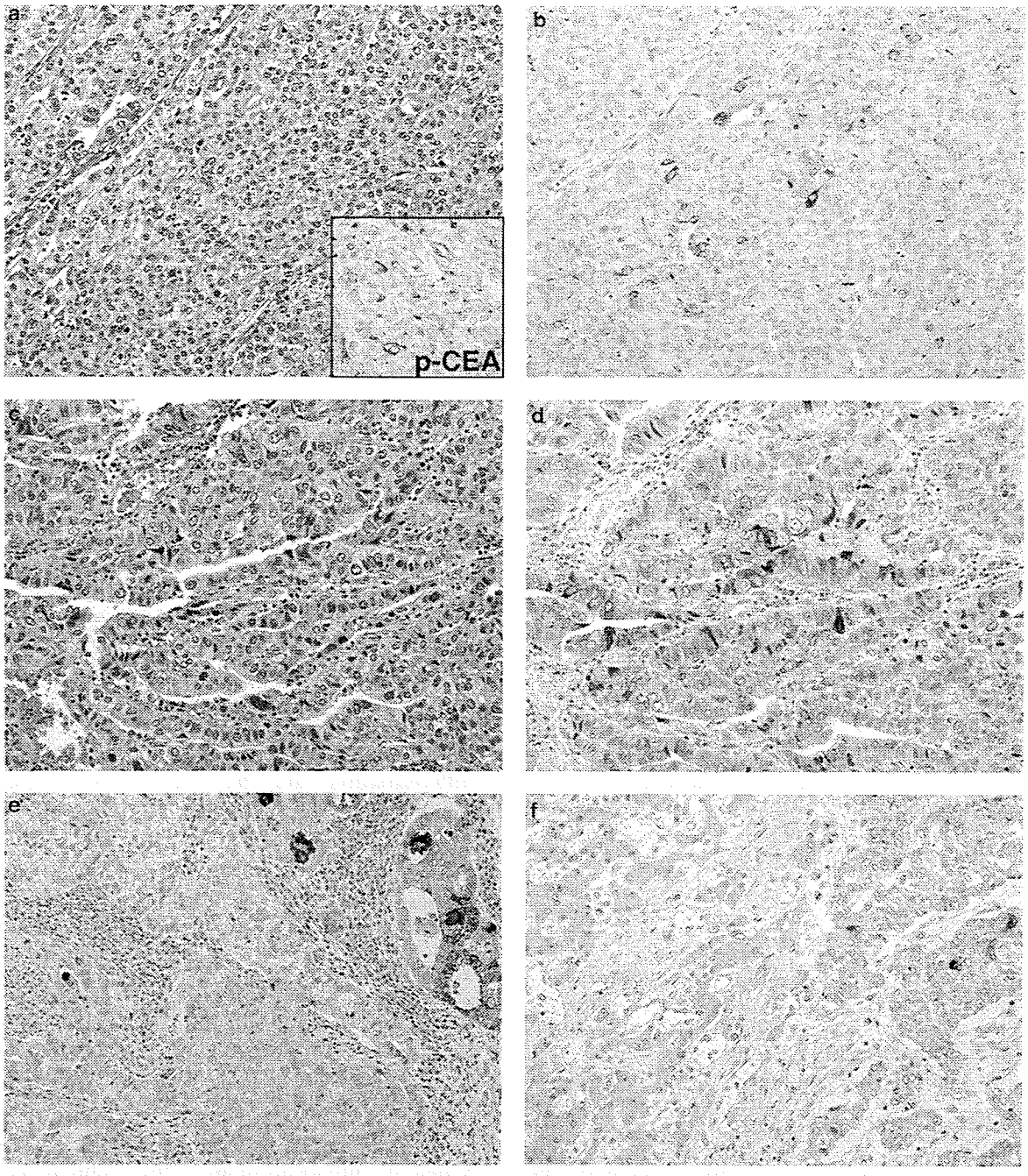

Figure 2 Immunostaining of PLUNC in hepatoid adenocarcinoma of the stomach. (**a**) H&E staining of hepatoid adenocarcinoma component of primary tumor. Tumor cells display a solid structure resembling that of hepatocellular carcinoma. Inset: hepatoid adenocarcinoma component showed a canalicular pattern. (**b**) Corresponding PLUNC staining. Focal-positive staining for PLUNC is observed. (**c**) H&E stain of tubular/papillary adenocarcinoma component of primary tumor that consists of polygonal tumor cells with large central nuclei. (**d**) Corresponding PLUNC staining. Focal-positive staining for PLUNC is observed. (**e**) The tubular adenocarcinoma component is stained by anti-PLUNC antibody, with luminal and cytoplasmic patterns, whereas the hepatoid adenocarcinoma component shows no staining. (**f**) Liver metastasis of gastric hepatoid adenocarcinoma. Focal-positive staining for PLUNC is detected, whereas adjacent non-neoplastic hepatocytes are not stained.

adenocarcinoma (Figure 4b). The two liver metastases of hepatoid adenocarcinoma contained less than 10% HepPar1-positive tumor cells. In contrast, most of the primary hepatocellular carcinoma cases (45 out of 52 cases, 87%) displayed strong and extensive staining (2+, 10 cases; 3+, 35 cases) of HepPar1 (Figure 4c). All six hepatoid adenocarcinoma cases showed CK19 staining (Figure 4d), and

Table 3 PLUNC, AFP, HepPar1, CK19, and CK20 immunoreactivity in hepatoid adenocarcinoma of the stomach

Case no.	*Organ*	*Hepatoid adenocarcinoma component*					*Tubular/papillary adenocarcinoma component*				
		PLUNC	*AFP*	*HepPar1*	*CK19*	*CK20*	*PLUNC*	*AFP*	*HepPar1*	*CK19*	*CK20*
52	Stomach	1+	2+	1+	2+	0	3+	0	1+	2+	0
126	Stomach	1+	1+	1+	2+	0	3+	0	0	3+	0
141	Stomach	1+	1+	0	1+	1+	1+	0	0	1+	0
142	Stomach	1+	1+	0	2+	0	2+	0	0	2+	1+
143	Stomach[a]	1+	2+	1+	1+	0	0	0	1+	1+	0
	Liver	1+	2+	1+	2+	0	—[b]	—	—	—	—
144	Stomach[a]	1+	2+	1+	2+	1+	1+	0	0	3+	0
	Liver	2+	3+	1+	3+	1+	—	—	—	—	—

0 indicates negative; 1+, ≤10%; 2+, 11–50%; 3+, >50%.

[a]Primary tumor.

[b]Tubular/papillary adenocarcinoma component is not found.

CK19 staining was observed in both liver metastases (Figure 4e). One case showed 80% CK19-positive tumor cells, and another case contained 20% CK19-positive tumor cells within the metastatic tumor. Forty-four out of 52 (85%) hepatocellular carcinoma cases showed no staining of CK19, and CK19 staining was observed in eight hepatocellular carcinoma cases (1+, 2 cases; 2+, 3 cases; 3+, 3 cases) (Figure 4f). Two out of six gastric hepatoid adenocarcinoma cases showed focal positivity for CK20, and both hepatoid adenocarcinoma cases showed less than 10% CK20-positive tumor cells (Figure 4g). CK20 staining was observed in one case of liver metastasis (Figure 4h). Forty-two out of 52 (81%) hepatocellular carcinoma cases showed no staining of CK20, and CK20 staining was observed in 10 hepatocellular carcinoma cases (1+, 3 cases; 2+, 4 cases; 3+, 3 cases) (Figure 4i).

Discussion

Gastric hepatoid adenocarcinoma frequently shows histologic features that mimic hepatocellular carcinoma. It can be very challenging to differentiate hepatoid adenocarcinoma from hepatocellular carcinoma on the basis of morphology alone, especially when a specimen is limited, such as small tissue biopsy specimens. Immunoreactivity for AFP, alpha-1 antitrypsin,[30] and Glypican 3[31] in hepatoid adenocarcinoma has been reported. Moreover, albumin mRNA has also been detected in hepatoid adenocarcinoma by *in situ* hybridization.[32] These molecules are expressed in normal adult or fetal liver and show various degrees of utility in distinguishing gastric hepatoid adenocarcinoma from conventional gastric cancer; however, these molecules are also expressed in hepatocellular carcinoma. Therefore, there is no reliable biomarker to distinguish hepatoid adenocarcinoma from hepatocellular carcinoma. In this study, we observed overexpression of PLUNC in gastric cancer with two comprehensive gene expression profiling methods, SAGE and microarray. Although only 7% of conventional gastric cancer cases showed focal immunostaining of PLUNC, extensive staining of PLUNC was observed in all six cases of hepatoid adenocarcinoma. Hepatocellular carcinoma cases did not show staining of PLUNC, whereas both cases of liver metastases of hepatoid adenocarcinoma were positive for PLUNC. These results indicate that PLUNC is a good marker to distinguish hepatoid adenocarcinoma from hepatocellular carcinoma. In this study, staining of PLUNC was not detected in normal adult and fetal liver. In gastric hepatoid adenocarcinoma, PLUNC-positive tumor cells were not stained by AFP. These findings led us to speculate that PLUNC is not involved in hepatocyte differentiation and that this is the reason why immunostaining of PLUNC can distinguish gastric hepatoid adenocarcinoma from hepatocellular carcinoma.

What are the unique features and utility of PLUNC immunostaining? It should be mentioned here that all the samples analyzed by immunostaining in the present study were surgically resected specimens. For surgical resection of a tumor, pathologic diagnosis from a biopsy specimen is essential. Because this is the first investigation of PLUNC in gastric cancer, we analyzed surgically resected specimens to investigate the precise distributions of PLUNC-positive cells. In this study, PLUNC was preferentially expressed in the tubular/papillary adenocarcinoma components of primary tumors. In gastric hepatoid adenocarcinoma, the tubular/papillary adenocarcinoma component tends to be located superficially in the primary tumor, whereas the hepatoid adenocarcinoma component tends to be located in the deeper parts of the primary tumor.[27,33] In fact, PLUNC-positive tumor cells were frequently found in superficial areas of gastric hepatoid adenocarcinoma. Therefore, PLUNC immunostaining can be used to test gastric biopsy specimens because only superficial areas are obtained by gastric biopsy. Furthermore, among 138 conventional gastric cancer cases, less than 1% of tumor cells were stained in nine gastric cancer cases, and only one gastric cancer case (P208T) showed

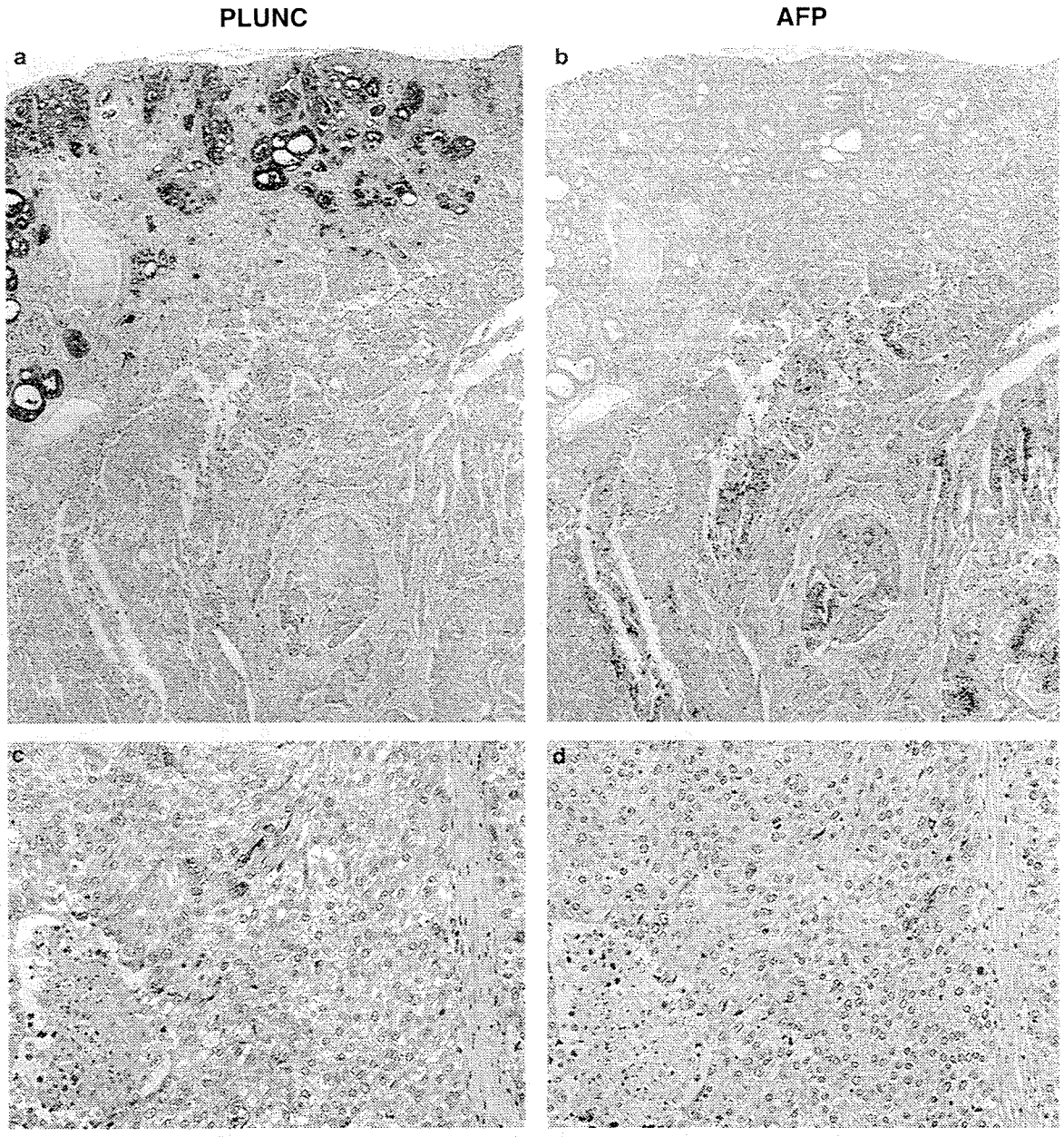

Figure 3 Immunostaining of PLUNC and AFP in hepatoid adenocarcinoma of the stomach. PLUNC staining is observed in the tubular adenocarcinoma component (**a**), whereas AFP staining is found in the hepatoid adenocarcinoma component (**b**). In the hepatoid adenocarcinoma component, PLUNC-positive tumor cells (**c**) are negative for AFP (**d**).

extensive staining of PLUNC. These results indicate that immunostaining of PLUNC can differentiate gastric hepatoid adenocarcinoma from conventional gastric cancer. Because PLUNC is frequently stained in the tubular/papillary adenocarcinoma component of gastric hepatoid adenocarcinoma, PLUNC staining can assist in diagnosing gastric hepatoid adenocarcinoma even when the hepatoid adenocarcinoma component is not found in gastric biopsy specimens.

The liver is the most common organ for metastasis by gastric hepatoid adenocarcinoma. It is difficult in some cases to distinguish a liver metastasis of hepatoid adenocarcinoma from a primary hepatocellular carcinoma with liver biopsy specimens because gastric hepatoid adenocarcinoma frequently shows histologic and immunostaining features that mimic those of hepatocellular carcinoma. It was previously reported that immunostaining for HepPar1, CK19, or CK20 is useful to distinguish gastric hepatoid adenocarcinoma from primary hepatocellular carcinoma.[27] In fact, most primary hepatocellular carcinoma showed extensive staining of HepPar1, whereas only focal staining of HepPar1 was observed in gastric hepatoid adenocarcinoma in

Table 4 Summary of PLUNC, AFP, HepPar1, CK19, and CK20 immunostaining

	No. of positive[a] cases		
	Primary gastric hepatoid adenocarcinoma (n = 6)	*Liver metastasis of gastric hepatoid adenocarcinoma (n = 2)*	*Hepatocellular carcinoma (n = 52)*
PLUNC	6 (100%)	2 (100%)	0 (0%)
AFP	6 (100%)	2 (100%)	11 (21%)
HepPar1	4 (67%)	2 (100%)	48 (92%)
CK19	6 (100%)	2 (100%)	8 (15%)
CK20	2 (33%)	1 (50%)	10 (19%)

[a]1+, 2+, and 3+ cases were considered positive.

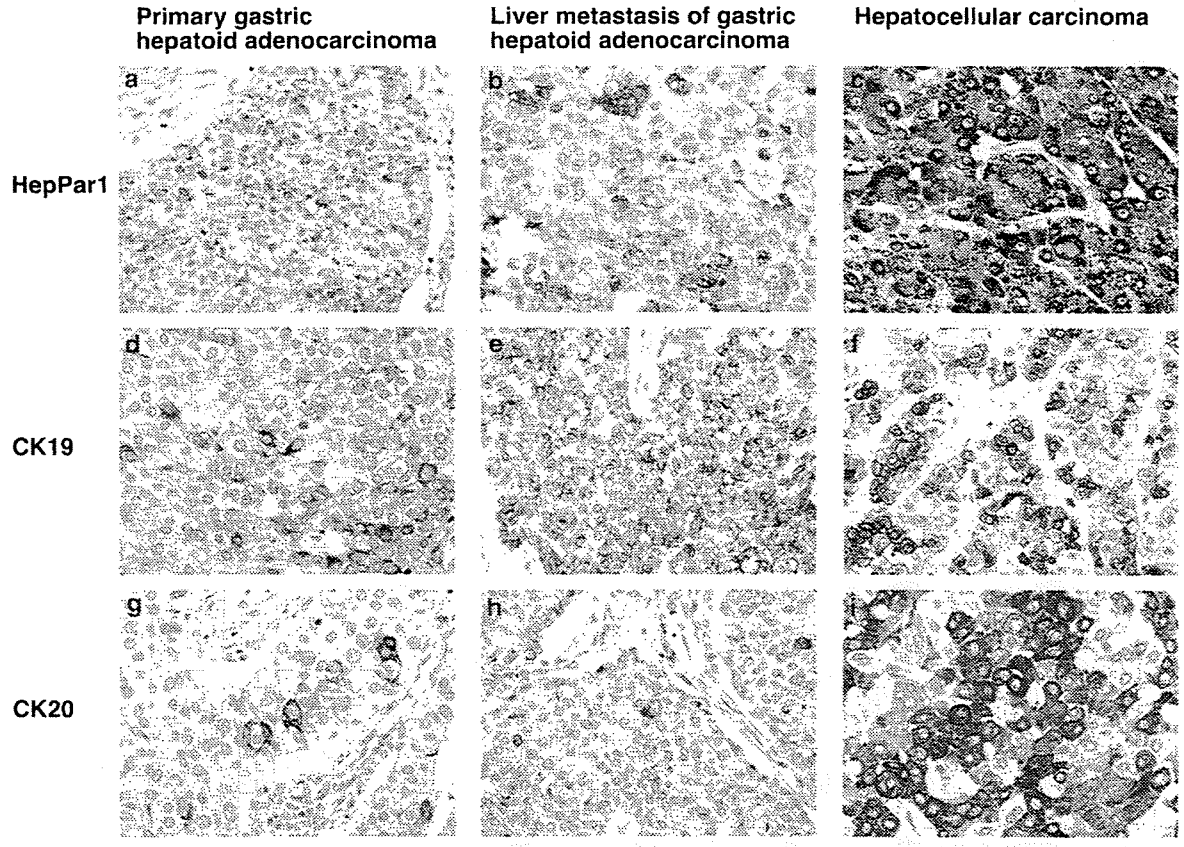

Figure 4 Immunostaining of HepPar1 (**a–c**), CK19 (**d–f**), and CK20 (**g–i**) in primary gastric hepatoid adenocarcinoma, liver metastasis of gastric hepatoid adenocarcinoma, and hepatocellular carcinoma. (**a**) In gastric hepatoid adenocarcinoma, hepatoid adenocarcinoma component shows focal-positive for HepPar1 within the primary tumor. (**b**) In liver metastasis of gastric hepatoid adenocarcinoma, focal-positive staining for HepPar1 is found. (**c**) Hepatocellular carcinoma cases show strong and extensive staining for HepPar1. (**d**) Cytoplasmic staining of CK19 is detected in hepatoid adenocarcinoma component in primary gastric hepatoid adenocarcinoma. (**e**) Diffuse CK19 staining is observed in liver metastasis of gastric hepatoid adenocarcinoma. (**f**) Some hepatocellular carcinoma cases show focal CK19 staining. (**g**) Focal CK20 staining is found in primary gastric hepatoid adenocarcinoma. (**h**) In liver metastasis of gastric hepatoid adenocarcinoma, focal-positive staining for CK20 is detected. (**i**) Some hepatocellular carcinoma cases show focal CK20 staining.

this study. Staining for CK19 and CK20 was detected more frequently in gastric hepatoid adenocarcinoma than in hepatocellular carcinoma in the present study. Because only six gastric hepatoid adenocarcinoma cases and two liver metastases of hepatoid adenocarcinoma were investigated in this study, it is difficult to conclude which is the better marker. At least however, because PLUNC staining was found only in gastric hepatoid adenocarcinoma, but not in hepatocellular carcinoma, PLUNC is a specific marker to distinguish hepatoid adenocarcinoma from hepatocellular carcinoma.

In this study, the 20 genes showing the greatest increase in expression on microarray were quite

different from those obtained by SAGE. Investigation of the difference between microarray and SAGE is beyond the scope of this study and will be described elsewhere.

In summary, we found that PLUNC is expressed in gastric hepatoid adenocarcinoma but not primary hepatocellular carcinoma. Although larger trials are required, this initial study shows the potential of PLUNC immunostaining to serve as a marker to distinguish metastatic hepatoid adenocarcinoma from primary hepatocellular carcinoma. In conventional gastric cancer cases, patients with PLUNC-positive gastric cancer had a significantly worse survival rate than those patients with PLUNC-negative gastric cancer; therefore, expression of PLUNC may be a key factor mediating the malignant behavior of gastric hepatoid adenocarcinoma.

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Immunohistochemical Staining of Reg IV and Claudin-18 is Useful in the Diagnosis of Gastrointestinal Signet Ring Cell Carcinoma

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Abstract: Signet-ring cell carcinoma (SRCC) is a unique subtype of adenocarcinoma that is characterized by abundant intracellular mucin accumulation and a crescent-shaped nucleus displaced toward one end of the cell. Identification of an SRCC's primary site is important for better planning of patient management because the treatment and prognosis differs markedly depending on the origin of the SRCC. In the present study, we analyzed the immunohistochemical characteristics of 94 cases of SRCC, including 21 cases of gastric SRCC, 16 of colorectal SRCC, 10 of breast SRCC, and 47 of pulmonary SRCC, with antibodies against Reg IV and claudin-18, which we previously identified as gastric cancer-related genes. We also tested known markers cytokeratin 7, cytokeratin 20, MUC2, MUC5AC, caudal-related homeobox gene 2 (CDX2), thyroid transcription factor-1, mammaglobin, gross cystic disease fluid protein 15, and estrogen receptor. All 21 cases of gastric SRCC and 16 cases of colorectal SRCC were positive for Reg IV, and the remaining SRCCs were negative. Eighteen of 21 (86%) gastric SRCCs and 6 of 16 (38%) colorectal SRCCs were positive for claudin-18, whereas another SRCCs were negative. In conclusion, Reg IV staining and claudin-18 staining can aid in diagnosis of gastrointestinal SRCC.

Key Words: signet-ring cell carcinoma, Reg IV, claudin-18, gastric cancer

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Signet-ring cell carcinoma (SRCC) is a unique subtype of adenocarcinoma that is characterized by abundant intracellular mucin accumulation and a crescent-shaped nucleus displaced toward one end of the cell. SRCC can arise in almost every organ and can present with distant metastases. In general, the prognosis of patients with SRCC regardless of the site of origin is poor.[14,17,28,33,35,38] SRCCs are morphologically identical irrespective of the primary site of origin or metastatic status. In some cases, the primary site of origin may be difficult to determine even after complete clinical and radiologic workups. This is a clinically significant problem because without such information, proper treatment and determination of prognosis may be delayed. Immunohistochemical profiling may aid in directing the workup of metastatic SRCC of an unknown primary site, and several markers are known. SRCC of the prostate can be differentiated from that of other sites by expression of prostate-specific antigen.[18] Similarly, SRCC of the lung expresses sensitive and specific markers, such as thyroid transcription factor-1 (TTF-1), that may help differentiate lung carcinoma from other tumors.[21] However, these SRCCs are not as common as SRCC of the stomach, colorectum, and breast, which comprise more than 90% of SRCC tumors.[2,11,14] To date, there has been no report describing useful markers for differentiating these tumors.

We previously performed serial analysis of gene expression of 4 primary gastric cancers[24] and identified several gastric cancer-related genes.[3] Of these genes, we found that *regenerating islet-derived family member 4* (*REG4*, which encodes Reg IV) is a candidate gene for cancer-specific expression, at least in patients with gastric cancer. Expression of Reg IV is limited to stomach, small intestine, colon, and pancreas in normal tissues.[25]

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Immunohistochemical analysis revealed that Reg IV is expressed in gastric cancer, colorectal cancer, and pancreatic cancer, whereas lung cancers and breast cancers do not express Reg IV.[22,25] Reg IV is also expressed in gastric SRCC and colorectal SRCC.[26] Therefore, Reg IV may serve as a marker of digestive organ cancer. We also found that expression of claudin-18 is restricted to normal stomach and duodenum, and is not expressed in other normal tissues.[29] Immunohistochemical analysis of claudin-18 revealed that although expression of claudin-18 is down-regulated in several gastric cancers, claudin-18 is expressed in gastric SRCC, suggesting that claudin-18 may be a marker of cancers derived from stomach or duodenum. However, expression of claudin-18 has not been investigated in tumors other than gastric cancers.

In the present study, we analyzed the immunohistochemical characteristics of SRCCs from various organs using antibodies against Reg IV and claudin-18. We also examined expression of cytokeratin (CK) 7, CK20, MUC2, MUC5AC, caudal-related homeobox gene 2 (CDX2), TTF-1, mammaglobin, gross cystic disease fluid protein-15 (GCDFP15), and estrogen receptor (ER). The combined expression patterns of CK7 and CK20 have recently been extensively studied in various primary and metastatic carcinomas.[8,10] Mucin production such as MUC2 and MUC5AC is the biologic hallmark of SRCC. CDX2 is a homeobox gene that is expressed exclusively in normal intestinal epithelium and its neoplasms.[20] Mammaglobin, a mammary-specific member of the uteroglobin family, is known to be overexpressed in human breast cancer.[37] GCDFP15, which is a predominant secretory protein in various body fluids, including saliva, milk, and seminal plasma, is generally considered a relatively specific and somewhat sensitive marker of breast cancers.[4] ER is also reported to be expressed in 60% to 70% of breast cancers.[1] We found that several markers alone and in combination can differentiate the primary site of SRCCs.

MATERIALS AND METHODS

Tissue Samples

We selected 54 cases of SRCC, including 21 cases of gastric SRCC, 16 of colorectal SRCC, 10 of breast SRCC, and 7 of pulmonary SRCC, from the surgical pathology files of the Hiroshima University Hospital or affiliated hospitals. Cases were selected randomly, and gastric SRCCs and colorectal SRCCs for which depth of invasion did not exceed the submucosal layer were excluded because Krukenberg tumors, which are considered a representative metastatic neoplasm, arise from a late-stage gastrointestinal tract neoplasm, in particular from gastric SRCC except few reports.[15] Cases with a known history of other malignancies were excluded. To qualify as SRCC, more than 50% of the examined tumor cells had to be signet-ring cells. Because written informed consent was not obtained, identifying information was removed from all samples before analysis to protect the privacy of all patients. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government.

Surgically resected specimens were routinely fixed in 10% buffered formalin and examined macroscopically. All sections contained tumor tissue and surrounding non-neoplastic tissues and were embedded in paraffin. Additional consecutive 5μm sections were cut from a selected tissue block and stained with hematoxylin and eosin. We also examined lung SRCC samples on a tissue microarray (TMA), which was provided by the National Cancer Center Hospital East (Chiba, Japan). TMAs were constructed according to a previously described protocol.[34] The study specimens were routinely oversampled with 2 replicate core samples of tumor (different areas). Each tissue-array block contained 40 cases of pulmonary SRCC.

Immunohistochemistry

A Dako Envision Kit (Dako, Carpinteria, CA) was used for immunohistochemical analysis of all markers. In brief, sections were pretreated by microwaving (500 W) in citrate buffer (pH 6.0) for 15 minutes to retrieve antigenicity. After endogenous peroxidase activity was blocked with 3% H_2O_2-methanol for 10 minutes, sections were incubated with normal goat serum (Dako) for 20 minutes to block nonspecific antibody binding sites. Sections were then incubated with the following primary antibodies (Table 1): anti-Reg IV, anticlaudin-18, anti-MUC2, anti-MUC5AC, anti-CDX2, anti-CK7, anti-CK20, anti-TTF-1, antimammaglobin, anti-GCDFP15, and anti-ER. Suppliers and working dilutions are noted in Table 1. Rabbit polyclonal anti-Reg IV antibody was raised in our laboratory.[25] The specificity of the Reg IV antibody has been characterized in detail.[25] Sections were incubated with primary antibody for 1 hour at 25°C, followed by incubations with peroxidase-labeled antirabbit or mouse IgG for 60 minutes. Staining was completed with a 10-minute incubation with the substrate-chromogen solution. The sections were counterstained with 0.1% hematoxylin. Appropriate positive and negative control samples were used.

TABLE 1. Antibodies Used in the Current Study

Antibody	Clone	Dilution	Pretreatment	Source
Reg IV	Polyclonal	1:50	MW	*
Claudin-18	Polyclonal	1:50	MW	Zymed Laboratories
MUC2	Ccp58	1:50	MW	Novocastra
MUC5AC	CLH2	1:50	MW	Novocastra
CK7	OV-TL 12/30	1:50	MW	DAKO
CK20	Ks20	1:50	MW	DAKO
CDX2	CDX2-88	1:20	MW	BioGenex
TTF-1	SPT24	1:50	MW	Novocastra
Mammaglobin	304-1A5	1:50	MW	DAKO
GCDFP15	23A3	1:50	MW	Novocastra
ER	6F11	Diluted	MW	VENTANA

*Rabbit polyclonal anti-Reg IV antibody was raised in our laboratory.
MW indicates microwaving (500 W) in citrate buffer (pH 6.0) for 15 min.

Evaluation of Positive Cases and Cutoff-point Thresholds

Immunostaining was evaluated independently by 2 investigators (K.S., and N.O.), and when the evaluations differed, a decision was made by consensus while investigators reviewed the specimen with a multihead microscope. Neoplastic tissue was evaluated semiquantitatively at magnifications of × 100 and × 400. Cytoplasmic immunoreactivity for CK7, CK20, MUC2, MUC5AC, mammaglobin, GCDFP15, and Reg IV; nuclear immunoreactivity for CDX2, TTF-1, and ER; and membranous reactivity for claudin-18 were assessed.

For surgically resected specimens, immunoreactivity was judged on the basis of the percentage of tumor cells expressing a particular antigen in each specimen. For the TMAs, staining was considered positive if any tumor cells were stained appropriately. The percentage of reactive cells necessary for a positive result reflects the viewpoint and opinion of the authors. There can be significant methodologic differences between studies and aware of the potential effect of these differences on a study's results. The aim of the present study was to differentiate SRCCs arising from various organs. Therefore, the cutoff-point for antibody reactivity necessary to define a result as positive was staining of any (> 0%) cells in both surgically resected specimens and TMAs.

RESULTS

Staining Patterns of Gastric SRCCs

Results of immunostaining of 21 gastric SRCCs are detailed in Table 2. Images are shown in Figures 1A to C. All cases (21/21) of gastric SRCCs expressed Reg IV. The percentage of Reg IV-positive tumor cells ranged from 1% to 90%. Cytoplasmic staining of Reg IV was considered positive. Of 21 gastric SRCCs, 18 (86%) showed membranous staining for claudin-18. Approximately 50% to 80% of gastric SRCCs expressed MUC2, MUC5AC, CK7, and CK20. Fourteen (67%) cases showed heterogenous CDX2 staining, and none expressed TTF-1, mammaglobin, GCDFP15, or ER.

Staining Patterns of Colorectal SRCCs

Detailed results for the immunostaining of 16 colorectal SRCCs (14 colorectum, and 2 appendix) are given in Table 2. Images are shown in Figures 1D to I. All 16 cases of colorectal SRCC expressed Reg IV. Like gastric SRCC, membranous immunostaining of claudin-18 was observed in 6 colorectal SRCCs. MUC2, CK20, and CDX2 were expressed in more than 80% of colorectal SRCCs. Six (38%) cases were positive for MUC5AC, and 2 (12%) cases were positive for CK7. No cases expressed TTF-1, mammaglobin, GCDFP15, or ER.

Staining Patterns of Pulmonary SRCCs

Detailed immunostaining results for 47 pulmonary SRCCs (7 surgically resected specimens, and 40 cases on TMA) are given in Tables 2 and 3. Images are shown in Figures 2A to D. None of the pulmonary SRCCs expressed Reg IV or claudin-18. TTF-1 was expressed in 42 (89%) pulmonary SRCCs (all 7 surgically resected specimens, and 35 of 40 TMA specimens). All pulmonary SRCCs were positive for CK7 and negative for CK20. Fewer than 50% of pulmonary SRCCs showed cytoplasmic staining of MUC2 and MUC5AC. CDX2, mammaglobin, GCDFP15, and ER were not expressed by pulmonary SRCCs.

Staining Patterns of Breast SRCCs

Detailed immunohistochemical staining data for 10 breast SRCCs are given in Table 2. Images are shown in Figures 2E to I. Neither Reg IV nor claudin-18 was expressed by breast SRCCs. Cytoplasmic expression of mammaglobin was observed in all 10 breast SRCCs. Nine (90%) of 10 breast SRCCs showed cytoplasmic staining of GCDFP15, and nuclear staining of ER. All 10 cases expressed CK7. Three (30%) of 10 breast SRCCs showed cytoplasmic staining of MUC2, whereas 1 (10%) breast SRCC expressed MUC5AC. None expressed CK20, CDX2, or TTF-1.

Summary of Immunostaining for Reg IV, Claudin-18, MUC2, MUC5AC, CK7, CK20, CDX2, TTF-1, Mammaglobin, GCDFP15, and ER

The aim of the present study was to distinguish SRCCs arising from various organs. Therefore, the cutoff-point for antibody reactivity for a positive result was defined as staining of any cells (> 0%) in both surgically resected specimens and TMA specimens. The results of immunostaining are given in Table 4. All 21 gastric SRCCs and 16 colorectal SRCCs expressed Reg IV. The remaining SRCCs were negative for Reg IV. Eighteen (86%) of 21 gastric SRCCs and 6 (38%) of 16 colorectal SRCCs were positive for claudin-18 expression, whereas other SRCCs were negative. Fourteen cases of 21 (67%) gastric SRCCs and 14 (88%) colorectal SRCCs were positive for CDX2. The remaining SRCCs were negative. Forty-two cases of 47 (89%) pulmonary SRCCs were positive for TTF-1, whereas SRCCs derived from other organs did not express TTF-1. All cases of breast SRCCs were positive for mammaglobin, whereas the remaining SRCCs were negative. GCDFP15 was expressed in 9 (90%) of breast SRCCs, and ER staining was observed in 9 (90%) of breast SRCCs. Staining patterns of the other molecules, including MUC2, MUC5AC, CK7, and CK20, varied.

DISCUSSION

Identification of the primary sites of SRCCs, especially in cases of extensive tumor progression, is important for appropriate patient management because the treatment and prognosis of SRCCs from different tissues differ. However, determination of the site of origin is difficult, if not impossible, especially with biopsy material. Occasionally, metastases are the first manifestation of the disease, and this poses a diagnostic problem. Therefore, we examined various SRCC specimens to

TABLE 2. Staining Distribution for Reg IV, claudin-18, MUC2, MUC5AC, CK7, CK20, CDX2, TTF-1, Mammaglobin, GCDFP15, and ER in SRCC of Various Organs*

Case No.	Primary Site	Reg IV	Claudin-18	MUC2	MUC5AC	CK7	CK20	CDX2	TTF-1	Mammaglobin	GCDFP15	ER
1	Stomach	60	70	25	0	0	20	50	0	0	0	0
2	Stomach	80	3	80	15	10	80	20	0	0	0	0
3	Stomach	80	0	70	0	15	70	60	0	0	0	0
4	Stomach	70	10	90	20	15	70	80	0	0	0	0
5	Stomach	90	3	70	0	0	60	0	0	0	0	0
6	Stomach	10	70	0	0	25	0	20	0	0	0	0
7	Stomach	20	40	20	60	70	0	2	0	0	0	0
8	Stomach	70	30	20	0	20	0	40	0	0	0	0
9	Stomach	1	40	0	0	5	0	5	0	0	0	0
10	Stomach	70	40	0	0	25	10	0	0	0	0	0
11	Stomach	60	20	70	80	80	0	20	0	0	0	0
12	Stomach	80	5	60	0	30	5	5	0	0	0	0
13	Stomach	70	20	80	30	70	15	0	0	0	0	0
14	Stomach	3	0	0	10	60	0	0	0	0	0	0
15	Stomach	20	30	10	0	5	0	0	0	0	0	0
16	Stomach	3	90	0	70	30	0	70	0	0	0	0
17	Stomach	3	70	0	20	60	0	0	0	0	0	0
18	Stomach	5	60	5	20	60	0	0	0	0	0	0
19	Stomach	30	30	40	30	0	15	60	0	0	0	0
20	Stomach	1	0	90	0	0	3	40	0	0	0	0
21	Stomach	60	10	80	0	15	40	15	0	0	0	0
22	Colon	90	0	100	1	0	70	90	0	0	0	0
23	Colon	80	3	70	0	0	5	80	0	0	0	0
24	Colon	75	0	20	15	0	30	30	0	0	0	0
25	Colon	2	10	0	0	0	30	0	0	0	0	0
26	Colon	90	15	90	10	0	40	80	0	0	0	0
27	Colon	90	15	80	0	0	70	70	0	0	0	0
28	Colon	80	0	20	0	0	30	60	0	0	0	0
29	Colon	70	20	60	0	0	15	70	0	0	0	0
30	Colon	70	0	70	10	0	70	20	0	0	0	0
31	Colon	50	0	30	25	20	60	50	0	0	0	0
32	Colon	70	30	70	60	10	30	40	0	0	0	0
33	Colon	40	0	50	0	0	15	30	0	0	0	0
34	Colon	40	0	70	0	0	15	0	0	0	0	0
35	Rectum	80	0	80	0	0	50	60	0	0	0	0
36	Appendix	60	0	90	0	0	3	80	0	0	0	0
37	Appendix	5	0	40	0	0	10	20	0	0	0	0
38	Lung	0	0	1	0	60	0	0	60	0	0	0
39	Lung	0	0	0	0	100	0	0	40	0	0	0
40	Lung	0	0	0	0.5	100	0	0	70	0	0	0
41	Lung	0	0	0	2	100	0	0	40	0	0	0
42	Lung	0	0	0	5	100	0	0	80	0	0	0
43	Lung	0	0	1	80	100	0	0	40	0	0	0
44	Lung	0	0	0	20	100	0	0	90	0	0	0
45	Breast	0	0	80	0	60	0	0	0	3	60	80
46	Breast	0	0	0	0	60	0	0	0	80	30	60
47	Breast	0	0	0	0	100	0	0	0	10	20	5
48	Breast	0	0	0	0	80	0	0	0	5	40	50
49	Breast	0	0	10	0	90	0	0	0	10	10	20
50	Breast	0	0	0	0	80	0	0	0	60	70	70
51	Breast	0	0	0	0	80	0	0	0	5	15	10
52	Breast	0	0	20	60	90	0	0	0	70	40	90
53	Breast	0	0	0	0	90	0	0	0	3	90	0
54	Breast	0	0	0	0	90	0	0	0	80	0	80

*Data are the percentage of reactive cells in each tumor.

address the issue of whether immunohistochemistry could be useful for differential diagnosis of SRCC. In the present study, we found that Reg IV and claudin-18 are immunohistochemical markers of gastrointestinal SRCC. We also examined expression of CK7, CK20, MUC2, MUC5AC, CDX2, TTF-1, mammaglobin, GCDFP15, and ER, and found that mammaglobin is useful for detection of breast SRCC.

In the present study, all gastrointestinal SRCCs expressed Reg IV, whereas SRCCs from other organs, including lung and breast, did not express Reg IV. Therefore, staining for Reg IV is useful to identify SRCCs

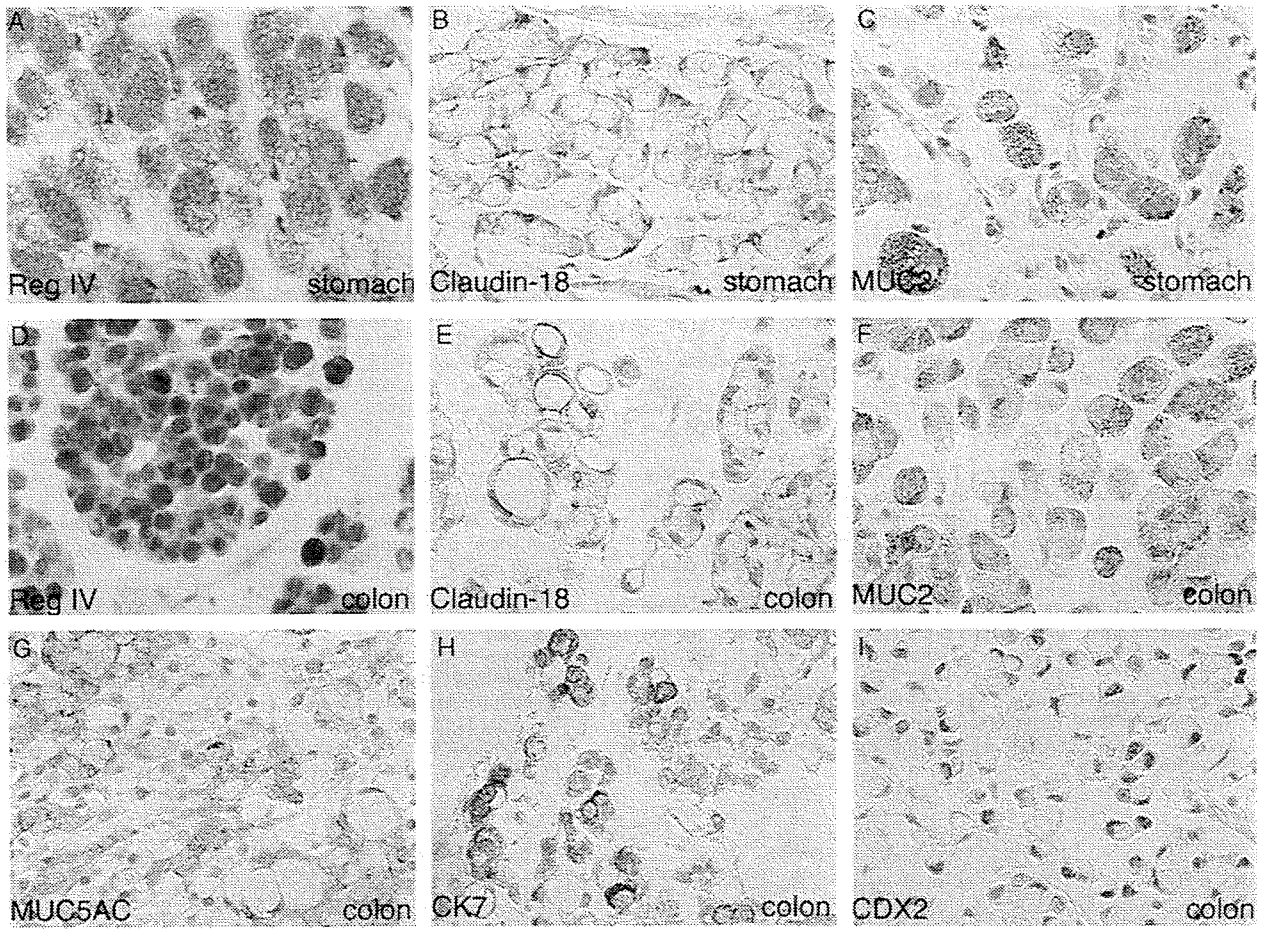

FIGURE 1. Immunohistochemical staining of digestive organ SRCCs [(A–C): gastric SRCC, (D–I): colorectal SRCC]. A and D, Reg IV immunoreactivity in the cytoplasm of SRCC (original magnification ×400). B and E, Membranous claudin-18 immunoreactivity (original magnification ×400). C and F, Cytoplasmic staining specific for MUC2 (original magnification ×400). G, Cytoplasmic staining of MUC5AC (original magnification ×400). H, Cytoplasmic staining of CK7 (original magnification ×400). I, Nuclear staining of CDX2 (original magnification ×400).

that originated from gastrointestinal sites. CDX2 is generally used as a marker of gastrointestinal tumors. However, CDX2 is expressed in 90% of gastric SRCCs and 89% of colorectal SRCCs.[9] Therefore, CDX2 is not always a reliable marker of gastrointestinal SRCC. In the present study, CDX2 expression was limited to gastrointestinal SRCCs; however, not all tumors were stained. The expression pattern of Reg IV resembles that of CDX2; however, Reg IV is expressed by 100% of gastrointestinal SRCCs.

TABLE 3. Frequency of Marker Staining in 40 Cases of Pulmonary SRCCs on TMAs*

Antibody	Positivity (%)
Reg IV	0 (0)
Claudin-18	0 (0)
MUC2	3 (8)
MUC5AC	11 (28)
CK7	40 (100)
CK20	0 (0)
CDX2	0 (0)
TTF-1	35 (88)
Mammaglobin	0 (0)
GCDFP15	0 (0)
ER	0 (0)

*Data are number of positive cases (%).

Claudin-18 was reported to be detected in gastric carcinoma, and is expressed in gastric SRCC.[29] Our current results show that claudin-18 expression is limited to gastrointestinal SRCC. Although claudin-18 was not expressed in 100% of gastrointestinal SRCCs, claudin-18 in combination with Reg IV may be a useful marker for detecting gastrointestinal SRCC and excluding other types of SRCC.

TTF-1 is generally considered to be a marker of differentiated alveolar lining cells. Pulmonary adenocarcinoma cells tend to express TTF-1, and studies have reported expression in more than 70% of cases.[19] TTF-1