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

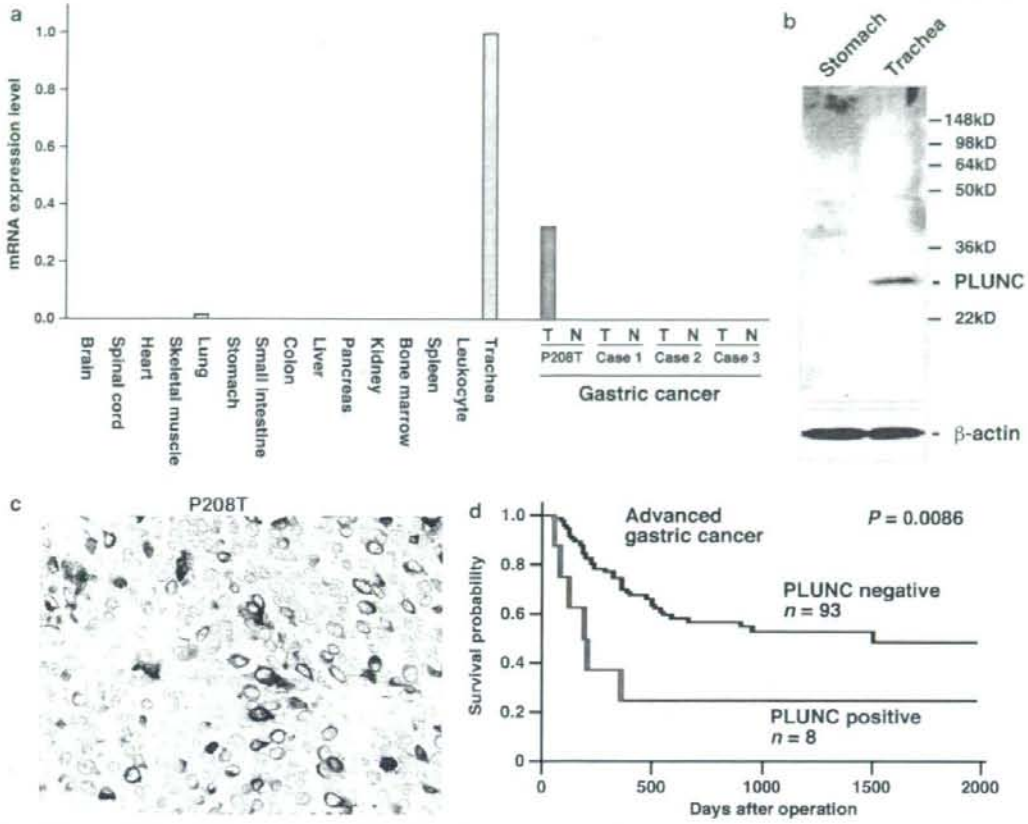

Figure 1 (**a**) Quantitative RT-PCR analysis of *PLUNC* in 15 normal tissues, four gastric cancer cases (T), and corresponding non-neoplastic mucosa (N). Case P208T was analyzed by SAGE and microarray. The units are arbitrary, and we calculated the level of *PLUNC* mRNA expression by standardization 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

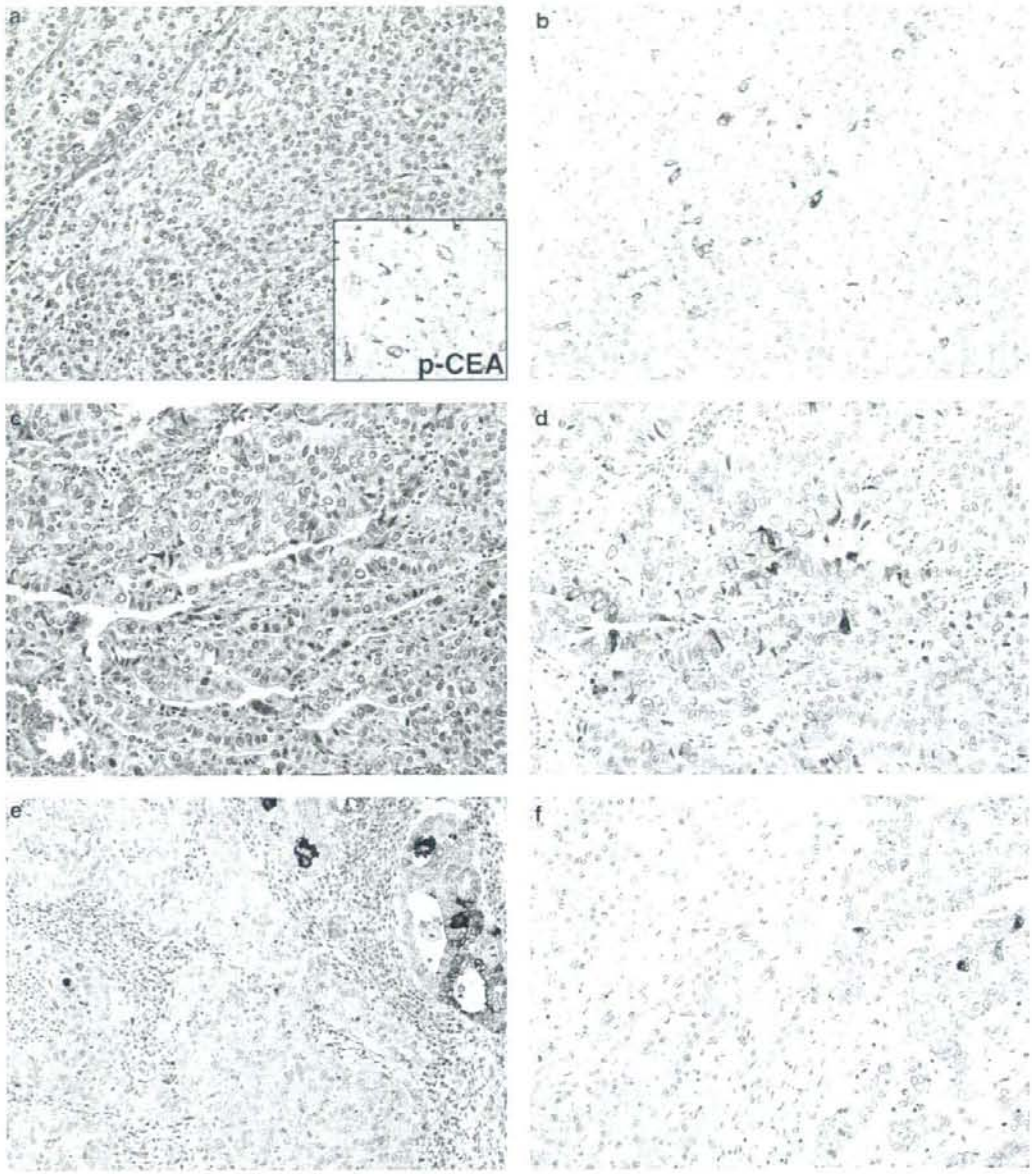

Figure 2 Immunostaining of PLUNC in hepatoid adenocarcinoma of the stomach. (**a**) H&E staining of hepatoid adenocarcinoma component of primary tumor. Tumor cells display a solid structure resembling that of hepatocellular carcinoma. Inset: hepatoid adenocarcinoma component showed a canalicular pattern. (**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

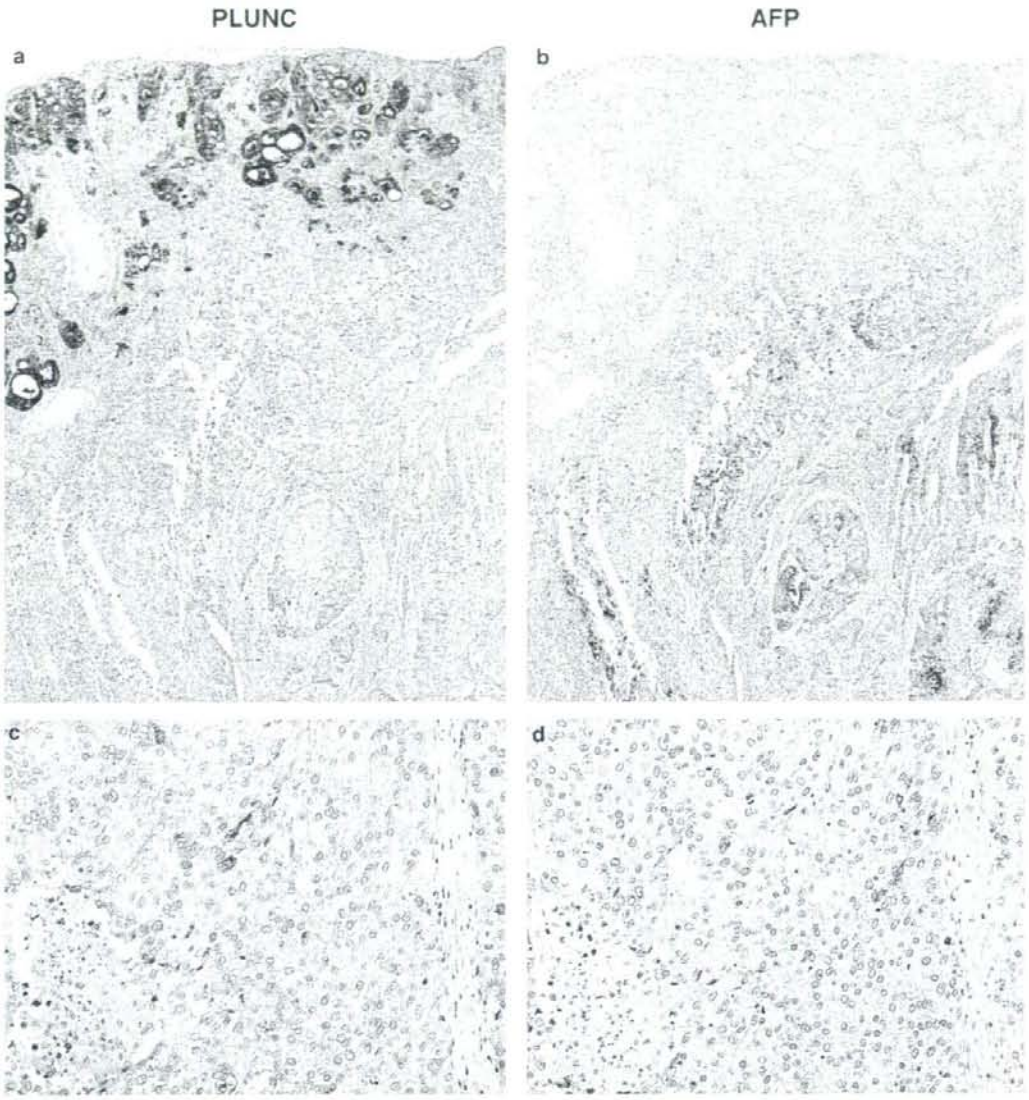

Figure 3 Immunostaining of PLUNC and AFP in hepatoid adenocarcinoma of the stomach. PLUNC staining is observed in the tubular adenocarcinoma component (**a**), whereas AFP staining is found in the hepatoid adenocarcinoma component (**b**). In the hepatoid adenocarcinoma component, PLUNC-positive tumor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

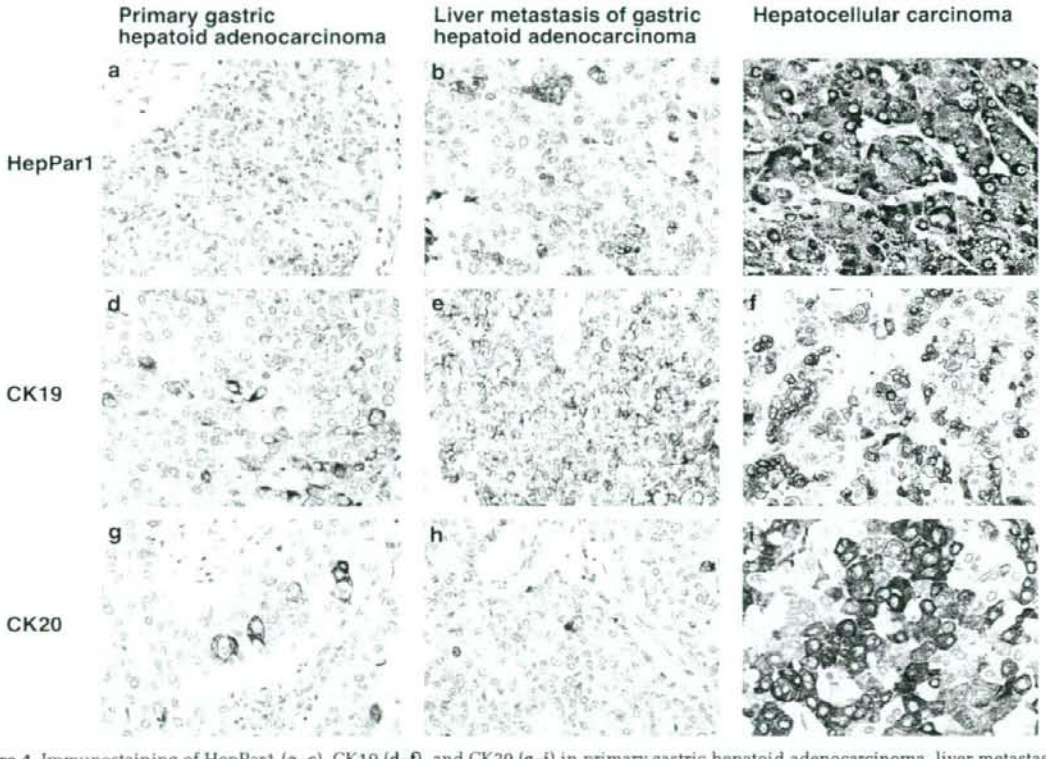

Figure 4 Immunostaining of HepPar1 (**a–c**), CK19 (**d–f**), and CK20 (**g–i**) in primary gastric hepatoid adenocarcinoma, liver metastasis of gastric hepatoid adenocarcinoma, and hepatocellular carcinoma. (**a**) In gastric hepatoid adenocarcinoma, hepatoid adenocarcinoma component shows focal-positive 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 nonneoplastic 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.

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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

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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

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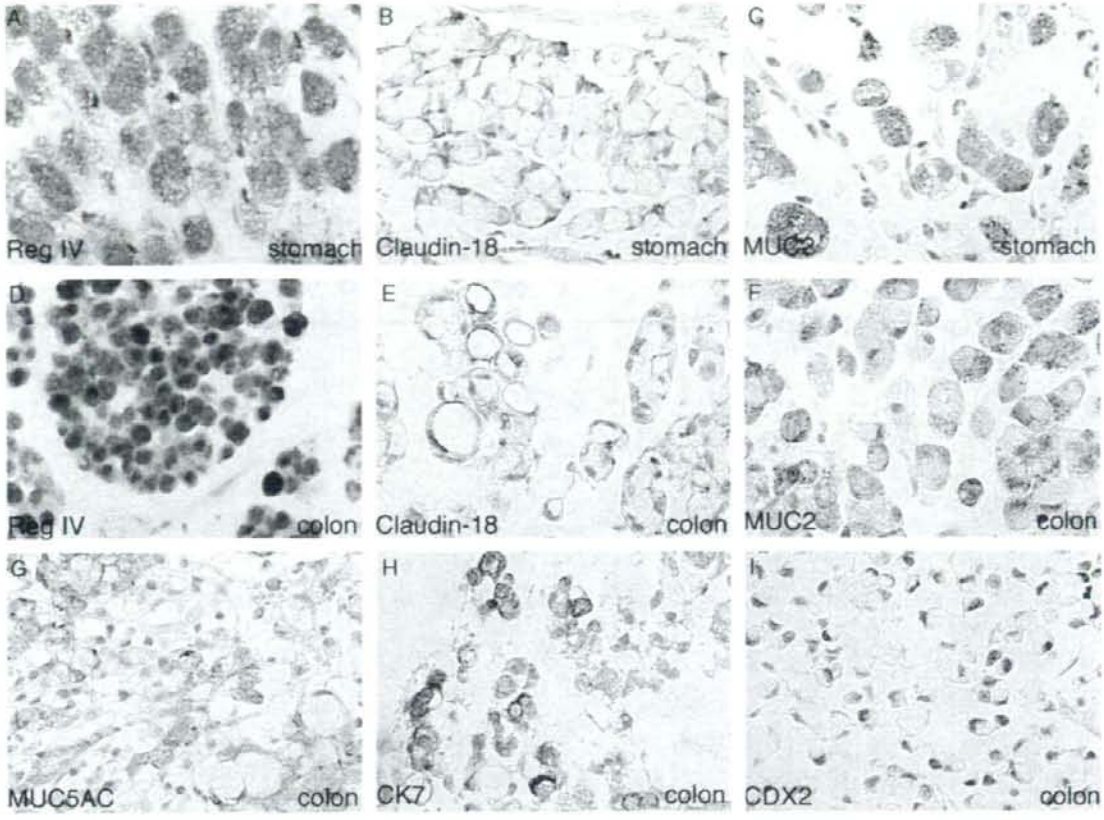

FIGURE 1. Immunohistochemical staining of digestive organ SRCCs [(A–C): gastric SRCC, (D–I): colorectal SRCC]. A and D, Reg IV immunoreactivity in the cytoplasm of SRCC (original magnification ×400). B and E, Membranous claudin-18 immunoreactivity (original magnification ×400). C and F, Cytoplasmic staining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

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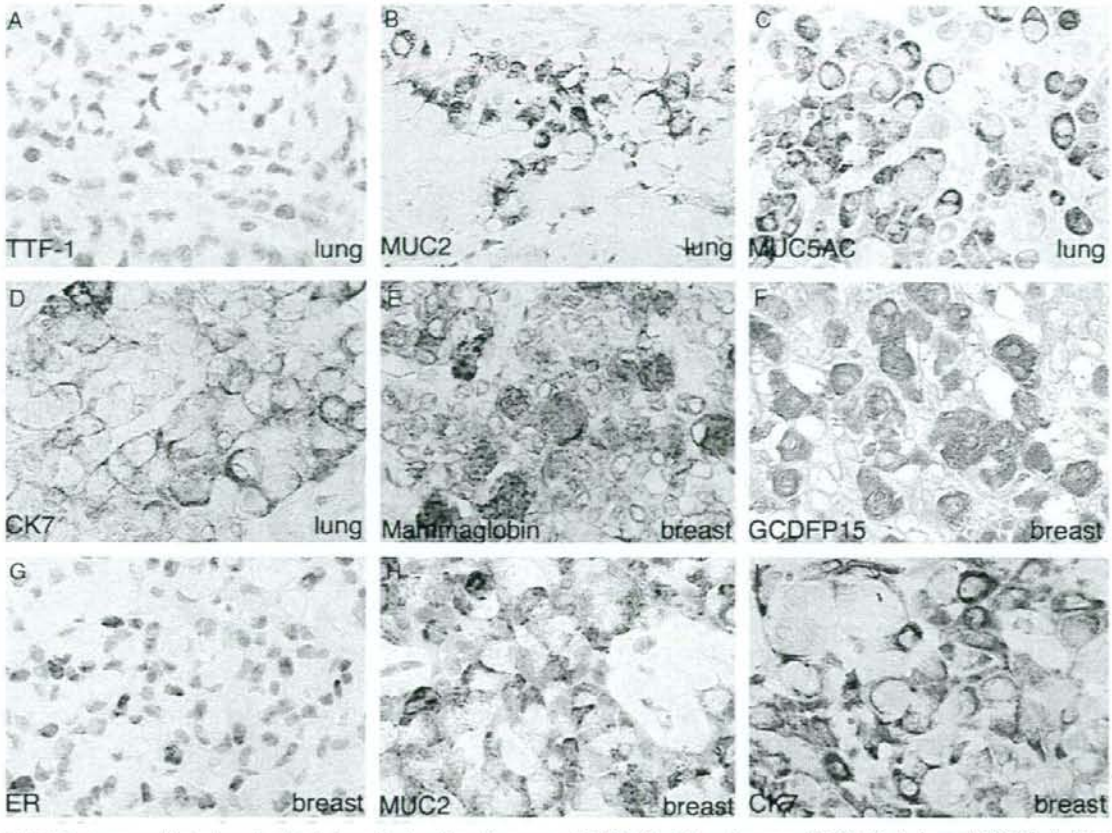

FIGURE 2. Immunohistochemical staining of extradigestive organ SRCCs [(A–D): pulmonary SRCC, (E–I): breast SRCC]. A, Tumor cells in a pulmonary SRCC show nuclear staining of TTF-1 (original magnification × 400). B and H, Cytoplasmic staining of MUC2 in a lung or breast (original magnification × 400). C, Cytoplasmic staining of MUC5AC in a lung SRCC (original magnification × 400). D and I, Cytoplasmic staining of CK7 in a lung or breast SRCC (original magnification × 400). E, Tumor cells in a breast SRCC show cytoplasmic staining of mammaglobin (original magnification × 400). F, Cytoplasmic staining of GCDFP15 in a breast SRCC (original magnification × 400). G, Nuclear staining of ER (original magnification × 400).

expression rates of 81%[34] to 100%[7] have been reported for SRCC of the lung. In the present study, TTF-1 was expressed in 89% of pulmonary SRCCs, and all other SRCCs were negative. Therefore, TTF-1–positive cases of SRCC are likely of pulmonary origin.

Mammaglobin is expressed in mammary tissue and primary breast tumors[36]; however, it is also reported to be expressed in carcinomas other than breast cancers[13] and in the secretory coil of the eccrine sweat glands of human skin.[30] Mammaglobin expression was detected immunohistochemically in 81 of 100 breast tumors independent of stage and histologic type.[37] To date, the pattern of expression of mammaglobin in SRCCs had not been studied, and this is the first report of immunohistochemical analysis of mammaglobin in SRCC. In the present study, mammaglobin was expressed in all cases of breast SRCC, but not in other SRCCs. Therefore, mammaglobin may be a useful marker to differentiate breast SRCC from other SRCCs.

GCDFP15 expression rates in breast cancer have been reported to range from 43% to 77%.[5,12,16,31] GCDFP15 was reported to be expressed in 80% of breast SRCC.[27] In the present study, GCDFP15 was expressed in 90% of breast SRCC, and all other SRCCs were negative. GCDFP15 was reported to be a more specific marker than mammaglobin for breast cancer, but it does not come up to the sensitivity of mammaglobin.[4]

In the present study, ER was expressed in 90% of breast SRCC, and all other SRCCs were negative. Although ER is expressed exclusively in breast carcinoma, approximately 20% of breast SRCC is negative for ER,[32] whereas some studies have found that up to 30% of gastrointestinal adenocarcinomas were positive for ER.[6,39] Thus, it is possible that immunohistochemical analysis

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TABLE 4. Summary of Positive Staining for Reg IV, Claudin-18, MUC2, MUC5AC, CK7, CK20, CDX2, TTF-1, Mammaglobin, GCDFP15, and ER in Various SRCCs*

Antibody	Stomach (n = 21)	Colorectum (n = 16)	Lung (n = 47)	Breast (n = 10)
Reg IV	21 (100)	16 (100)	0 (0)	0 (0)
Claudin-18	18 (86)	6 (38)	0 (0)	0 (0)
MUC2	15 (71)	15 (94)	5 (11)	3 (30)
MUC5AC	10 (48)	6 (38)	16 (34)	1 (10)
CK7+/ CK20+	7 (33)	2 (12)	0 (0)	0 (0)
CK7+/ CK20−	10 (48)	0 (0)	47 (100)	10 (100)
CK7−/ CK20+	4 (19)	14 (88)	0 (0)	0 (0)
CK7−/ CK20−	0 (0)	0 (0)	0 (0)	0 (0)
CDX2	14 (67)	14 (88)	0 (0)	0 (0)
TTF-1	0 (0)	0 (0)	42 (89)	0 (0)
Mammaglobin	0 (0)	0 (0)	0 (0)	10 (100)
GCDFP15	0 (0)	0 (0)	0 (0)	9 (90)
ER	0 (0)	0 (0)	0 (0)	9 (90)

*Data are number of positive cases (%).

for ER alone might not distinguish breast SRCC from gastrointestinal SRCC.

The expression patterns of CK7, CK20, MUC2, and MUC5AC were not sufficiently unique to differentiate completely SRCC primary sites. The combined expression patterns of CK7 and CK20 have been studied extensively in various primary and metastatic carcinomas.[8,10] In the present study, the CK7-negative, CK20-positive expression pattern was observed in most of colorectal SRCCs, whereas gastric SRCCs had no fixed pattern. Our results revealed that breast SRCCs were CK7-positive and CK20-negative. Tsuta et al[34] found that pulmonary SRCCs are CK7-positive and CK20-negative. In the present study, all pulmonary SRCCs expressed CK7 but not CK20, similar to breast SRCC. These results suggest that the combined CK7 and CK20 expression pattern is useful to predict some SRCC primary sites; however, the specificity is low. MUC2 is an intestinal mucin and MUC5AC is a gastric mucin. Both are secreted. MUC2 and MUC5AC can be used as markers of gastric and colorectal SRCCs.[9,23] In the present study, MUC2 and MUC5AC were expressed with variable proportions of each tumor. The specificity of MUC2 and MUC5AC expression pattern was low.

In conclusion, we found that Reg IV staining and claudin-18 staining can aid in the diagnosis of gastrointestinal SRCC. Larger trials are needed to confirm these results. To differentiate SRCC primary sites, we propose that immunohistochemical panels that include Reg IV, claudin-18, TTF-1, and mammaglobin should be used. Staining for Reg IV is useful to identify SRCCs originating from the gastrointestinal tract. Claudin-18 can differentiate gastrointestinal SRCCs, TTF-1 can differentiate pulmonary SRCCs, and mammaglobin can distinguish mammary SRCCs.

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Overexpression of *RegIV* in Peritoneal Dissemination of Gastric Cancer and Its Potential as A Novel Marker for the Detection of Peritoneal Micrometastasis

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Abstract. *Background: Regenerating gene type IV (RegIV) is a candidate marker for cancer and inflammatory bowel disease. In this study, its potential as a novel marker for the detection of gastric cancer peritoneal micrometastases was examined. Patients and Methods: RegIV mRNA levels in the peritoneal washes of 95 gastric cancer patients and 22 with benign disease were quantified by real-time RT-PCR. To examine whether expression of RegIV enhance tumorigenicity or not, thirty two mice were injected intraperitoneally or subcutaneously with RegIV transfectants of TMK-1 cells, parental TMK-1 cells, or neomycin control transfectants. Results: RegIV expression was markedly higher in patients with peritoneal metastases compared to those without. The level of RegIV mRNA in gastric cancer patients was related to the extent of wall penetration. A cut-off value for RegIV-positive expression was based on an analysis of negative control patients with benign disease, and gastric cancer patients above the cut-off value constituted the micrometastasis (MM+) group. Based on this criteria, 3 out of 43 T1 or T2 cases were MM+ (93% specificity). Among 15 patients with peritoneal dissemination (7 out of 15 cases were positive by cytology), 14 cases were positive for RegIV expression (93% sensitivity), while analysis of carcinoembryonic antigen (CEA) mRNA failed to detect micrometastases in 4 cases (73% sensitivity). Combined analysis of CEA and RegIV improved the accuracy of diagnosis to 100%. The prognosis of RegIV-positive cases was significantly worse than that of RegIV-negative cases. Multivariate analysis using the Cox proportional hazards model suggested that RegIV may be an independent prognostic factor. Stable expression of RegIV significantly enhanced peritoneal metastasis in an animal model of gastric cancer. Conclusion: These findings suggest that RegIV mRNA expression has the potential to serve as a novel marker for detecting peritoneal dissemination in gastric cancer.*

Gastric cancer is the most common malignancy of the gastrointestinal tract in Japan and in certain Southeast Asian populations, and the second most common cause of cancer-related deaths in the world (1). The prognosis of patients with gastric cancer that has invaded the gastric serosa is poor, with a 5-year survival rate of less than 35% (2). In such cases, peritoneal dissemination is reported to be the most frequent type of recurrence after curative resection (3, 4). Free cancer cells derived from serosal invasion may be an indicator of early peritoneal seeding with subsequent

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Abbreviations: RT-PCR: Reverse transcriptase-polymerase chain reaction; CY: cytology; MM: micrometastasis; *RegIV*: regenerating gene type IV.

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formation of metastatic colonies. Thus, their detection represents a potentially valuable predictor of outcome for patients with advanced gastric cancers (5, 6). Cytological examination of peritoneal washes from laparotomies to detect free metastatic cancer cells has been used to evaluate the risk of recurrent disease (6, 7). Conventional cytology, however, lacks sufficient sensitivity, as some patients with negative cytology results have presented with recurrence in the form of peritoneal dissemination (5). Carcinoembryonic antigen (*CEA*)-specific RT-PCR has been used to detect cancer cells in peritoneal fluids (8); however, the results indicate that *CEA* expression is not 100% accurate as a marker, suggesting that more reliable markers are needed.

Previously, we examined global differential gene expression in gastric cancer cell lines established from a primary tumor and from metastases to the peritoneal cavity (9). Using a high-density cDNA microarray, we analyzed the expression of approximately 21,168 genes. The results of this study revealed that 24 genes were up-regulated and 17 genes were down-regulated in gastric cancer cell lines established from metastases to the peritoneal cavity. One of the up-regulated genes was *RegIV*. *RegIV* is a member of the regenerating gene (Reg) family, which is part of the larger calcium-dependent lectin (C-type) gene superfamily (10). Reg family members are a group of small secretory proteins which can function as acute phase reactants, lectins, antiapototic factors, or growth factors for pancreatic β cells, neural cells and epithelial cells in the digestive tract (11). The Reg family proteins also play an important role in the injury response in the gastrointestinal mucosa. *RegIV* expression is up-regulated in response to mucosal injury in active Crohn's disease and ulcerative colitis, and is increased in most colorectal cancers compared to normal tissue (10, 12-14). Recently, *RegIV* expression in gastric cancer was reported and was found to be closely related to the infiltrating potential of the carcinoma (15-17). In one study, RT-PCR analysis was used to show a high level of *RegIV* expression in gastric cancer (16). However, while overexpression of *RegIV* in gastric cancer has been reported, the role of RegIV in gastric cancer peritoneal dissemination has not been investigated. In this study, amplification of *RegIV* by quantitative RT-PCR from peritoneal lavage cells was used to develop a highly sensitive method for detecting micrometastases of cancer cells. This detection method has the potential to predict peritoneal recurrence in gastric cancer patients with a higher level of accuracy than previous methods.

Patients and Methods

Cell culture. The gastric cancer cell lines SNU-1, SNU-5, SNU-16, and SNU-719 were established previously by Park *et al.* (18). The mesothelial cell line Met5A was established by Duncan *et al.* (19, 20). The gastric cancer cell lines KATO-III and GT3TKB, and the acute myeloid leukemia cell line HL60 were purchased from Riken Cell Bank (Tsukuba, Japan). Another gastric cancer cell line, TMK-1 was kindly donated by Professor Tahara, of Hiroshima University. Cells were maintained at 37˚C in a humidified atmosphere of 5% CO_2 in high-glucose RPMI-1640 (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, penicillin and streptomycin.

Clinical samples and peritoneal washes. Patients underwent surgery at Kyoto Prefectural University of Medicine between 1999 and 2004 and underwent regular postoperative surveillance for at least 2 years or until death. One patient who died within 30 days of surgery as a result of perioperative complications was excluded. The current study population consisted of 95 patients with gastric cancer and 22 with benign disease, such as cholelithiasis. Of the 95 patients with resectable cancer, 77 underwent potentially curative R0 resection; the remaining 18 were treated with palliative resection. The 95 cases included 21 patients with T1 tumors (tumor confined to the mucosa or invading as far as the submucosa), 22 with T2 tumors (invasion beyond the submucosa but not as far as the serosa), 37 with T3 tumors (serosal invasion), and 15 with T4 tumors (invasion to adjacent tissues). The population included 15 patients with synchronous peritoneal metastasis.

The peritoneal wash was collected from the Douglas cavity at laparotomy. Written informed consent was obtained from each patient prior to tissue acquisition. In the absence of ascites, 150 ml of saline was introduced into the Douglas cavity at the beginning of the operation and aspirated after general stirring. The washes were centrifuged at 2000 rpm for 10 minutes to collect intact cells, which were then rinsed with phosphate-buffered saline (PBS).

RNA preparation. Total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). The mRNA from each cell line was extracted using the FAST Track Kit Ver.2 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Northern blot analysis. Northern blot analysis was performed as described elsewhere (21-23). In brief, mRNA was prepared from each cell line, then fractionated on 1% agarose/2.2 M formaldehyde gels. Probes were labeled with ^{32}P by random priming. Each blot was hybridized with the *RegIV* probe and a β-actin probe as a control. Hybridization signals were analyzed with a BAS 2000 image analyzer (Fuji, Tokyo, Japan) and calculated the degree of overexpression in comparison to the β-actin control.

Real-time quantitative RT-PCR. cDNA was produced from 2 μg of total RNA using the Superscript Preamplification System (BRL, Bethesda, MD, USA), according to the manufacturer's instructions. Briefly, RNA was heated to 70˚C for 10 min in 14 μl of diethylpyrocarbonate-treated water containing 0.5 μg of oligo (dT) primer. Synthesis buffer (10x, 500 mM Tris-HCl, pH 8.3, 750 mM KCl, 30 mM $MgCl_2$), 2 μl of 10 mM dNTP mixture, 2 μl of 0.1 M DTT and reverse transcriptase (Superscript RT; 200U/μL, Gibco BRL, Gaithersburg, MD, USA) were added to the sample. The reaction mixture was incubated at 42˚C for 50 min, and the reaction was terminated by incubation at 90˚C for 5 min.

Quantitative PCR was performed using real time Taqman TM technology, as described by Nakanishi *et al.* (24). Results were analyzed on a Model 5700 Sequence Detector (Applied Biosystems Corp., Foster City, CA, USA).

The *RegIV* RT-PCR primers used were 5'-TCCTTGCAC TAGCTACATCC-3' and 5'-GGAATGTATGGCCCACATCA-3'. The *CEA* RT-PCR primers used were 5'-TCTGGAACT TCTCCTGGTCTCTCAGCTGG-3' and 5'-TGAAGCTGTTGCA

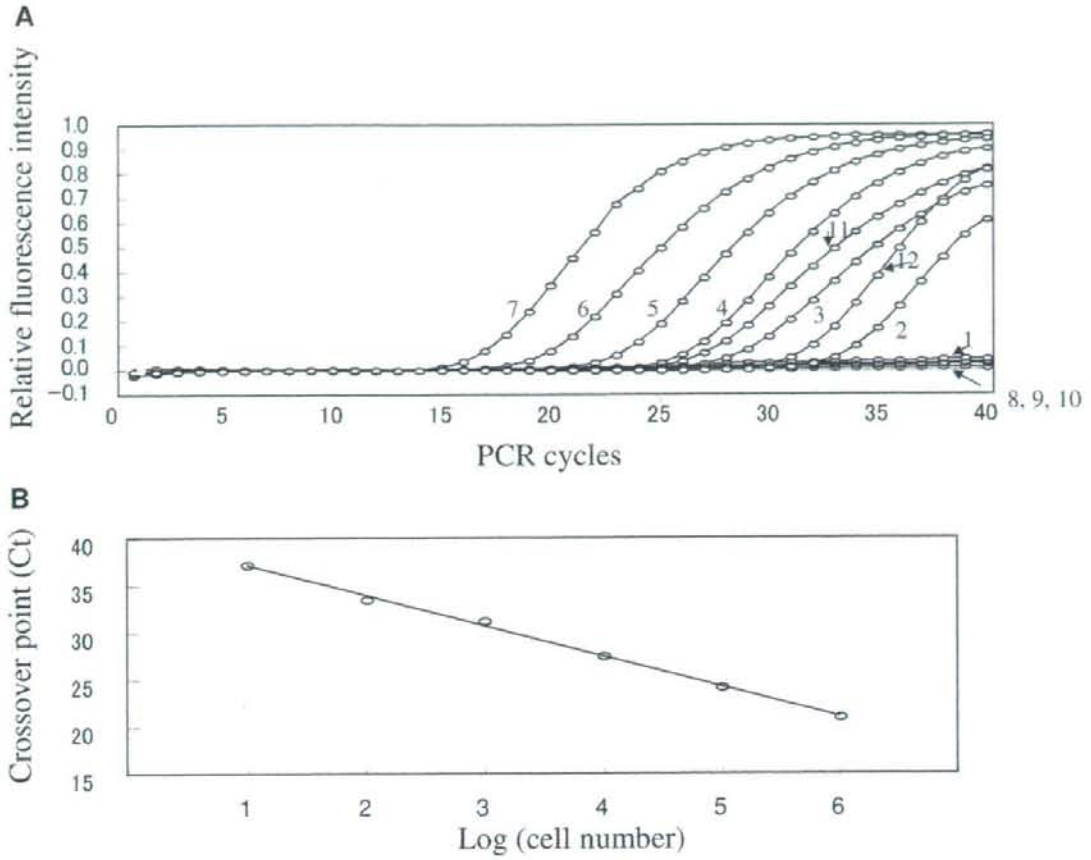

Figure 1. *Representative real-time RT-PCR result and calibration curve for estimating RegIV mRNA expression. A, Relative fluorescence vs. number of PCR cycles. Six external standards (lines 1-6) and two patient samples (lines 10 and 11) of unknown concentration were amplified with real-time 'Taqman TM' technology and analyzed with a Model 5700 Sequence Detector. Line 1=1, line 2=10, line 3=10^2, line 4=10^3, line 5=10^4, line 6=10^5, and line 7=10^6 SNU-16 gastric cancer cell equivalents of cDNA; line 8=Met 5A cells; line 9=HL60 cells; line 10=negative peritoneal wash; lines 11 and 12=positive peritoneal washes. B, Calibration curve for estimating RegIV mRNA expression. Curve was generated using data from the external controls in A by plotting the crossover points (Ct) against log SNU-16 cell number. Relative RegIV mRNA values in patient samples were calculated using this curve.*

AATGCTTTAAGGAAGAAGC-3'. Hybridization probes for detecting PCR products were labeled with a reporter dye (FAM), at the 5' end and a quenching dye (TAMRA), on the 3'end. The sequence of the *CEA* hybridization probe was 5'-(FAM) CATCTGGAACTTCTCCTGGTCTCTCAGC(TAMRA)-3'; the identification number for the hybridization probe for *RegIV* is Hs00230746 (Applied Biosystems Corp.).

For RT-PCR, the reaction mixture contained 1.25 units of Amp-Taq DNA polymerase, 1xPCR reaction buffer, 180 ng of each primer, 200 mM dNTP, 400 mM dUTP, 100 nM Taqman probe and 0.5 U Amplirase (Applied Biosystems Corp.). Serial dilutions of control cDNA were analyzed for each target gene. Crossover point (Ct) values were determined corresponding to the cycle number at which fluorescence emission reached a threshold standard deviation of ten above the mean baseline emission derived from 40 cycles. *CEA*- and *RegIV*-specific primers were used to generate standard curves from which the rate of change in the Ct value was determined for each patient sample (as shown in Figure 1). The cycling parameters were as follows: 2 min at 50°C, 10 min at 95°C, then 40 cycles of 15s at 95°C and 1 min at 60°C.

To minimize errors arising from variations in the amount of starting RNA among the samples, amplification of β-actin mRNA was analyzed as an internal reference. The values from target RNAs were then normalized to β-actin mRNA. The primers and the probe for β-actin were purchased from Applied Biosystems. Normalized results are expressed as the ratio of number of copies of target gene to β-actin.