the breast. In summary, Reg IV is a strong immunohistochemical marker for gastrointestinal signet-ring cell carcinoma, with a specificity and sensitivity of 100%.

PLUNC

Another example of a histopathological diagnostic marker identified through transcriptome dissection is PLUNC.[37] Of the technologies for high-throughput analysis of gene expression, the principles of microarray analysis and SAGE are totally different. Microarray analysis is based on hybridization, while SAGE is based on PCR and sequencing. Therefore, a list of upregulated or downregulated genes extracted using each method may be different. The lists of the 20 most upregulated genes detected on microarray analysis using GeneChip Human Genome U133Plus 2.0 array (Affimetrix, Santa Clara, CA, USA) and SAGE (GSM9103)[14] in the same sample of poorly differentiated adenocarcinoma of the stomach were compared. There were no genes that were the same among the top 20 differentially expressed genes that were extracted using the two methods. PLUNC was the gene showing the greatest increase in expression in gastric cancer on microarray analysis, but only three tags of PLUNC were detected on SAGE.

PLUNC was first cloned as a gene expressed in embryonic palate, nasal epithelium, and adult lung of the mouse by the differential display method.[38] Human PLUNC shows a similar expression pattern.[39] It is known that PLUNC encodes a secretion protein, and may participate in the early immune response against bacterial and viral infection in the upper respiratory tract, but the biological function of PLUNC remains poorly understood.[40] PLUNC is reported to be commonly expressed in adenocarcinoma, but not in small cell carcinoma and squamous cell carcinoma of the lung.[40,41] No information for PLUNC expression is available for other cancers. Quantitative RT-PCR showed that PLUNC expression was detected in adult trachea and lung, and one of the gastric cancers, while all other normal organs examined did not express PLUNC. Immunohistochemically, only 9% of gastric cancers were positive for PLUNC. Among those, cases with >30% of tumor cells stained were found to be hepatoid adenocarcinoma of the stomach (Fig. 6).

Gastric hepatoid adenocarcinoma is the primary gastric cancer producing AFP, and represents approximately 5% of all stomach cancers.[42] Liver metastasis is frequent and the prognosis is poor in comparison with ordinary gastric cancer.[43] Gastric hepatoid adenocarcinoma has histological features that mimic hepatocellular carcinoma, and discrimination is extremely difficult from hepatocellular carcinoma. It is challenging to differentiate hepatoid adenocarcinoma from hepatocellular carcinoma on the basis of morphology, especially if it metastasizes in the liver. So far there has been no marker

that can completely distinguish both. It was reported that immunohistochemical detection of HepPar1, CK19 or CK20 is useful for differentiating between the two.[44] HepPar1 staining is detected more frequently in hepatocellular carcinoma than in gastric hepatoid adenocarcinoma, whereas staining for CK19 and CK20 is detected more frequently in gastric hepatoid adenocarcinoma than in hepatocellular carcinoma. But many exceptions to this exist. Most of the gastric hepatoid adenocarcinomas in both primary and liver metastatic tumors were positive for PLUNC, whereas no HCC was PLUNC positive. Therefore, PLUNC is a novel and useful marker for differentiating gastric hepatoid adenocarcinoma from hepatocellular carcinoma.

ROLE OF THE NEWLY IDENTIFIED GENES IN DEVELOPMENT, DIFFERENTIATION AND PROGRESSION OF GASTRIC CANCER

Using transcriptome dissection based on SAGE, part of the molecular mechanism of development, differentiation and progression of gastric cancer has been clarified. Here, the connection with tumor differentiation is introduced. Gastric cancers are histologically classified into two major groups: differentiated and undifferentiated types, or intestinal and diffuse types based on glandular structure.[45,46] In addition, gastric cancers are also classified into intestinal (I) and gastric (G) types on the basis of the mucin expression profile such as expression of MUC2, CD10, MUC5AC, and MUC6.[47,48] CDX1 and CDX2 are intestine-specific transcription factors, and SOX2 is known to be stomach specific.[49,50] Some differences in molecular events have been noted: for instance, hypermethylation of the hMLH1 gene and microsatellite instability are frequent in the G type, and p53 mutation is frequent in the I type.[1,51,52] Also, with regard to biological difference, it has been suggested that the G type behaves more aggressively than the I type.[53]

Reg IV

As aforementioned, there are two staining patterns of Reg IV in gastric cancer: mucin-like staining and perinuclear staining. Mucin-like staining of Reg IV was significantly associated with MUC2-positive I type, and double staining demonstrated co-expression in the same tumor cells (Fig. 7a; Table 1).[21] In HT-29 colon cancer cells with forced expression of CDX2-ER, 4-hydroxytamoxifen (4-OHT) induces nuclear translocation and activates CDX2.[54] In this system CDX2 induced the expression of Reg IV. On immunofluorescent staining, all of the Reg IV-positive cells in both intestinal metaplasia and gastric cancer were also positive for CDX2, but CDX2-positive cells were not always Reg IV positive (Fig. 7b;
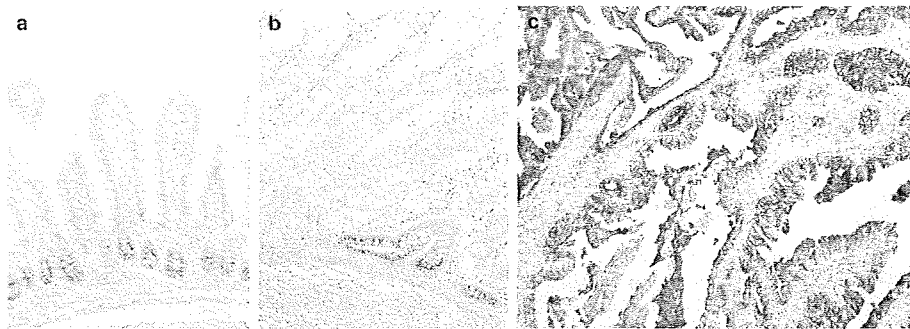
Figure 5 Immunostaining of GW112 in non-neoplastic tissues and gastric cancer. (a) GW112 is expressed in the cytoplasm of epithelial cells in the bottom area of the small intestine and (b) intestinal metaplasia of the stomach. (c) GW112 is strongly expressed in well-differentiated gastric cancer.
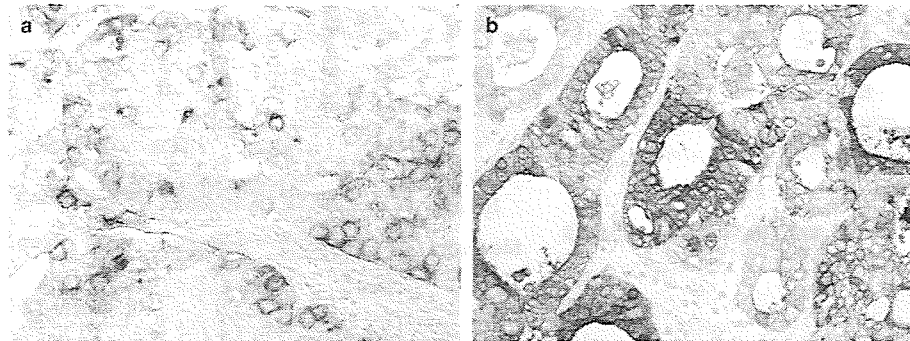

Figure 6 Immunostaining of palate, lung, and nasal epithelium carcinoma-associated protein (PLUNC) in hepatoid adenocarcinoma of the stomach. (a) Many tumor cells with a solid structure resembling hepatocellular carcinoma are positive for PLUNC. (b) Tumor cells in the tubular component of hepatoid adenocarcinoma are also positive for PLUNC.
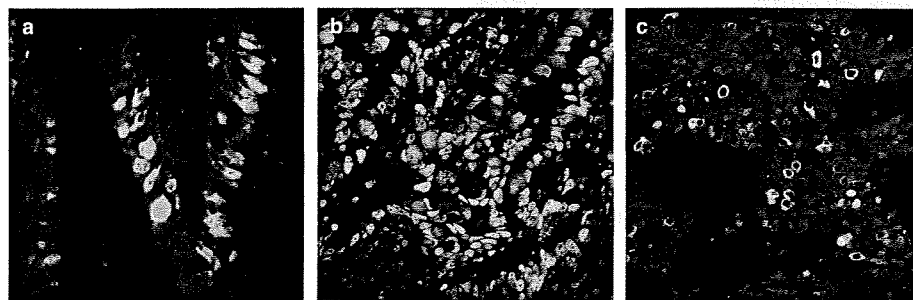

Figure 7 Relationship between regenerating islet-derived family, member 4 (Reg IV) and differentiation of gastric cancer. Double staining with immunofluorescent dyes shows co-expression of (a) Reg IV (red) and MUC2 (green) and (b) Reg IV (red), CDX2 (green) and 4'6'-diamidino-2-phenylindole (DAPI) (blue), and (c) co-localization of Reg IVs (red) and serotonin (green) in gastric cancer, respectively. CDX2, caudal-related homeobox gene 2; MUC, mucin; Reg IV, regenerating islet-derived family, member 4.

Table 1 Reg IV, MUC2, CDX2 and chromogranin A in gastric cancer on immunostaining

	Reg IV expression Positive	Fisher's exact test P value
MUC2		
Positive	40/52 (77%)	$P < 0.0001$
Negative	2/91 (2%)	
CDX2		
Positive	36/53 (68%)	$P < 0.0001$
Negative	6/90 (7%)	
Chromogranin A		
Positive	18/27 (67%)	$P < 0.0001$
Negative	24/116 (21%)	

CDX2, caudal-related homeobox gene 2; MUC, mucin; Reg IV, regenerating islet-derived family, member 4.

Table 1). These findings indicate that CDX2 is an upstream regulator for Reg IV expression.

The downstream targets of Reg IV are currently being searched for on GeneChip analysis, in combination with quantitative RT-PCR in *Reg IV*-transfected gastric cancer cell lines. SOX9 was found to be one of the downstream targets of Reg IV. Clear induction of SOX9 by Reg IV was detected in MKN-28 cells with an intestinal mucin phenotype. SOX9 is a member of the *sry*-related high-mobility group (HMG) box (SOX) family of HMG DNA-binding domain transcription factors, and is required for the development and differentiation of multiple cell lineages.[55] SOX9 is expressed in progenitor/stem cells and Paneth cells in the small intestine and is known to be regulated by the Wnt pathway.[56,57] Immu-

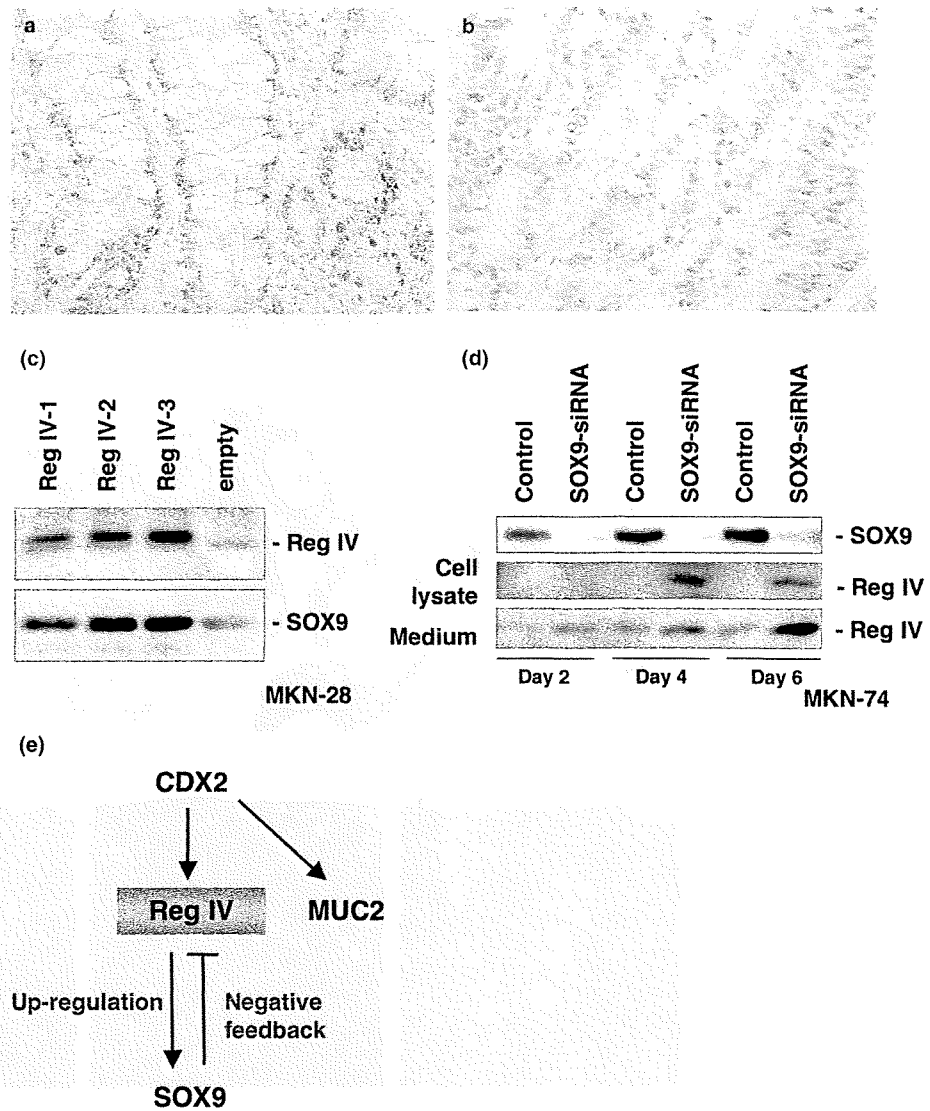**Figure 8** Expression of SOX9 and its regulation by regenerating islet-derived family, member 4 (Reg IV) in gastric cancer. (a) Immunostaining of SOX 9 in intestinal metaplasia of the stomach and (b) I type gastric cancer. (c) Forced expression of *Reg IV* in MKN-28 gastric cancer cells (Reg IV-1, -2, -3) induces SOX9, while (d) treatment of *SOX9*-siRNA to MKN-74 gastric cancer cells induces Reg IV in both cell lysate and culture medium on western blot. (e) Schematic illustration of the relationship between Reg IV and SOX9.

nohistochemically, SOX9 was expressed in epithelial cells at the proliferative zone of the normal gastric mucosa and bottom area of the intestinal metaplasia of the stomach (Fig. 8a). Many tumor cells of I type gastric cancer were positive for SOX9, and SOX9-positive tumor cells co-localized with *Reg IV*-positive cells. Furthermore, forced expression of Reg IV induced SOX9 in MKN-28 cells at the protein level, while addition of *SOX9*-siRNA to MKN-74 displaying a good level of SOX9 expression induced Reg IV expression (Fig. 8b). Therefore, a feedback mechanism might exist between Reg IV and SOX9 (Fig. 8c).

In contrast, perinuclear staining of Reg IV in gastric cancer indicated significant association with neuroendocrine differentiation monitored by the expression of chromogranin A and synaptophysin.[21] Using a tissue microarray with 630 cases of gastric cancer, connection of Reg IV with a variety of neuroendocrine hormones was examined, and it was found that Reg IV-positive cancers were significantly associated with serotonin- and gastrin-positive cancers. Furthermore, double immunofluorescent staining demonstrated that Reg IV was co-localized with serotonin (Fig. 7c; Table 1), gastrin, and pancreatic polypeptide in both cancer cells and non-neoplastic epithelial cells of the stomach.

Figure 9 summarizes the role of Reg IV and its pathway in gastric cancer. Reg IV may play a certain role in intestinal differentiation of gastric cancer through CDX2 and SOX9. Reg IV activates the EGFR and upregulates the anti-apoptotic signal, allows cells to escape from apoptosis, and participates in tumor growth and 5-FU resistance. The consequences of this association with endocrine differentiation, such as production of serotonin, and gastrin and its pathway, need further investigation.
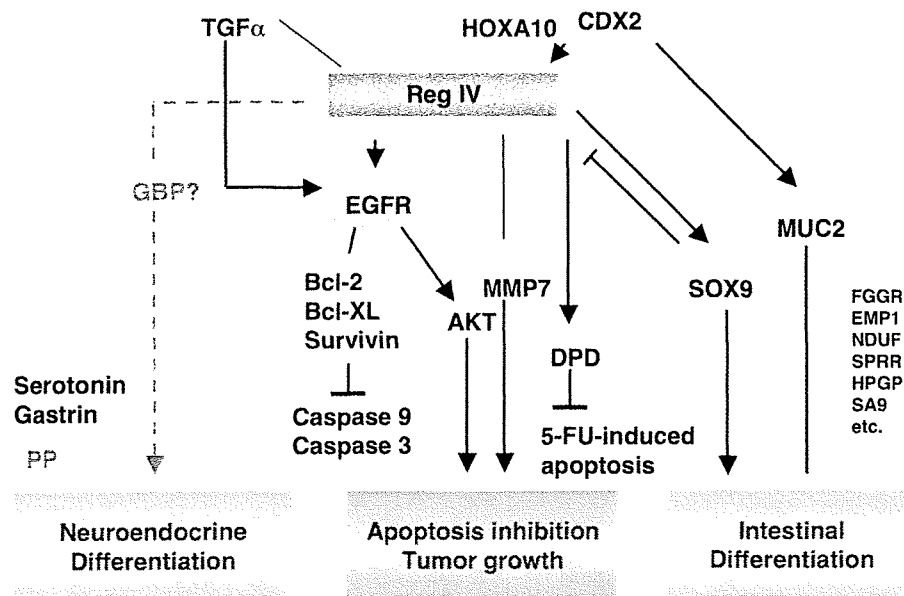
Figure 9   Role of regenerating islet-derived family, member 4 (Reg IV) and its pathway in gastric cancer.

HOXA10

Next several genes and molecules identified on transcriptome dissection are described, which relate to gastric cancer with intestinal differentiation.

Using the same method as that for identification of *PLUNC*, the lists of the 20 most upregulated genes detected on microarray analysis and SAGE (GSM8867)[14] were compared in a sample of well-differentiated gastric adenocarcinoma with an intestinal phenotype (I-type gastric cancer). Although only three genes were commonly detected as being upregulated on both methods, most of the upregulated genes extracted for each method were different. Among them, *HOXA10* had the greatest increase in expression in I-type gastric cancer on microarray analysis, whereas the tag for *HOXA10* was totally negative in SAGE.

HOXA10 is a member of the homeobox gene superfamily of transcription factors that contain a helix-turn-helix DNA-binding motif, and which participates in myeloid cell differentiation and proliferation.[58,59] HOXA10 and HOXA9 are associated with acute myeloid leukemia (*AML*) and mixed lineage leukemia (*MLL*) fusion genes.[60] HOXA10 controls uterine organogenesis during embryonic development and endometrial differentiation in adults.[61] Deregulation of HOXA10 correlates with progression of endometrial carcinoma.[62] Recently it was reported that CDX2 is an upstream regulator for *HOXA10* in myeloid cells and participates in leukemogenesis.[63] Quantitative RT-PCR indicated that 70% of gastric cancers overexpressed *HOXA10* regardless of tumor stage. On immunostaining HOXA10 was preferentially expressed in I-type gastric cancers and some of the intestinal metaplasias. The relation between HOXA10 and

MUC2 was statistically significant. Because knockdown of *HOXA10* by siRNA treatment in MKN-45 gastric cancer cells reduced expression of Reg IV, HOXA10 may be an upstream regulator for Reg IV. Although the role of HOXA10 in stomach carcinogenesis remains unknown, HOXA10 is a novel marker for I-type gastric cancer.

Claudin-18

During the course of the search for novel tumor suppressor genes on SAGE data analysis, another gene related to gastric cancer with an intestinal phenotype was identified. To identify genes showing decreased expression in gastric cancer, the gastric cancer SAGE libraries (GSM8505 and GSM8867)[14] were obtained from well-differentiated gastric cancers with an intestinal phenotype, and normal stomach SAGE library (GSM784). Tags from each gastric cancer library were compared with those of the normal stomach library, and with selected tags that were downregulated in gastric cancer libraries.[64] Among 25 genes thus selected, expression of *CLDN18* (encoding claudin-18) was found to be lost in six of the eight gastric cancer cell lines on conventional RT-PCR. Quantitative RT-PCR showed that expression of *CLDN18* was downregulated in approximately 60% of gastric cancer tissues.

Claudins are components of tight junction strands.[65] Tight junctions are localized at the most apical region of polarized epithelial cells and create a barrier to prevent paracellular transport and restrict the lateral diffusion of membrane lipids and proteins. The claudin protein family consists of 24 members (claudins 1–24) and are expressed in an organ-

Table 2 Results of case–control study for G/A genotype in the promotor of CLDN-18 variant 1

Genotype	Gastric cancer (n = 154)	Control (n = 304)	P-value	Crude OR (95% CI)
Age (maen ± SD)	63.4 ± 11.1	48.7 ± 20.8	0.00005	5.17
A/A	5 (3.2%)	45 (14.8%)		(2.01–13.3)
A/G	88 (57.2%)	133 (43.8%)		
G/G	61 (39.6%)	126 (41.4)		

The observed genotype distribution of controls was in agreement with Hardy–Weinberg equilibrium. Association was analyzed on Fisher's exact test. P are values for G/G+A/G genotypes relative to A/A genotype. OR are for G/G+A/G genotypes relative to A/A genotype.
CI, confidence interval; OR, odds ratio.

specific manner. Association between claudins and cancer has previously been suggested.[66] CLDN18 was first identified as a downstream target of the T/EBP/NKX2.1 homeodomain transcription factor.[67] There are two variants: variant 1 is expressed in the lung and variant 2 is expressed in the stomach.

RT-PCR in human samples confirmed that variant 1 was expressed in the lung and variant 2 was expressed in the stomach and small intestine, while other tissues including the colon, liver, pancreas and so on, did not express either variant.[64] More than 50% of gastric cancers had lost the expression of variant 2, whereas all samples of the corresponding non-neoplastic gastric mucosa expressed variant 2 at a good level. From immunostaining, claudin-18 was expressed on the cell membrane of all epithelial cells of the normal gastric mucosa, and Paneth cells of the duodenum.[64] Expression of claudin-18 was reduced in some of the intestinal metaplasia, most gastric adenomas, and approximately 60% of gastric cancers. In addition, advanced gastric cancer patients with reduced claudin-18 expression had poorer prognosis than those with preserved claudin-18 expression, while no association was found between claudin-18 expression and other clinicopathological characteristics. The analysis of mucin phenotype and claudin-18 expression demonstrated that reduced expression of claudin-18 was significantly associated with intestinal phenotype. A typical example is shown in Fig. 10. The expression of claudin-18 was lost in the MUC2-positive I type, while the MUC5AC-positive G type had preserved claudin-18 expression. Recently, it was reported that the protein kinase C (PKC)/mitogen-activated protein kinase (MAPK)/activator protein-1 (AP-1) dependent pathway regulated the expression of variant 2 of claudin-18 in gastric cancer cells.[68] While it is still necessary for a functional analysis to be performed, downregulation of claudin-18 may be involved in gastric cancer with an intestinal phenotype, and may participate in stomach carcinogenesis at an early stage, and also in progression of the cancer and poor survival rate for patients.

Polymorphism of cancer-related genes is known to affect cancer development and progression.[69] It is known that
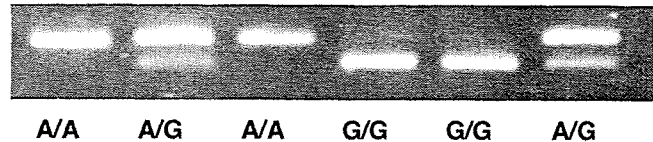

Figure 11 Polymerase chain reaction–restriction fragment length polymorphism analysis for G/A genotype in the promoter of CLDN-18 variant 1. See Table 2 for information on genotype distribution.

certain genetic polymorphisms also alter chemosensitivity and toxicity.[70] There is a single nucleotide polymorphism (SNP), guanine (G) to adenine (A) substitution at position −191 bp from the transcriptional start site within the promoter of CLDN-18 variant 1. This SNP was analyzed on PCR–restriction fragment length polymorphism in peripheral blood DNA samples taken from gastric cancer patients and control subjects under informed consent. The case–control study demonstrated that genotypes G/G and G/A were significantly more frequent in gastric cancer patients than in control subjects, and that the odds ratio of the G/G and G/A genotype then indicated a significantly enhanced risk for gastric cancer of 5.17 (95% confidence interval: 2.01–13.3) compared to the A/A genotype (Fig. 11; Table 2). This information could be directly connected with personalized cancer prevention planning.

CDH17

By comparing SAGE libraries between early and advanced gastric cancers of an intestinal phenotype (GSM8505 and GSM8867),[14] CDH17 was extracted as one of the most upregulated genes in advanced cancer.[71] CDH17 encodes cadherin-17, also called liver–intestine (LI)-cadherin, and is a structurally unique member of the cadherin superfamily.[72,73] Cadherin-17 has only 20 amino acids in the cytoplasmic domain, while classic cadherins have a highly conserved cytoplasmic domain that consists of 150–160 amino acids. CDH17 expression is regulated by CDX2 in the gastrointes-
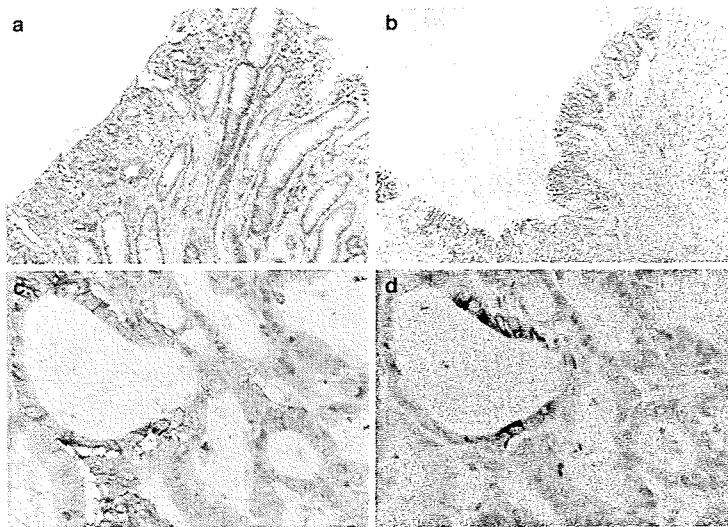
Figure 10   Immunostaining of claudin-18 in gastric cancer and its relation with mucin phenotype. (a,b) Claudin-18 expression is lost in MUC2-positive I-type gastric cancer, while (c,d) MUC5AC-positive G type had preserved claudin-18 expression. (a,c, claudin-18; b, MUC2; d, MUC5AC.)
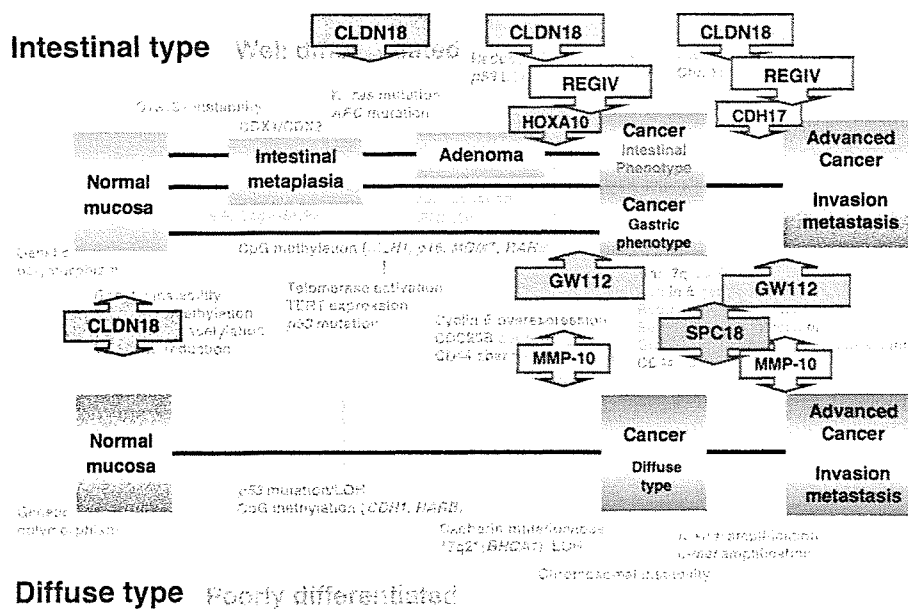

Figure 12   Role of novel genes and molecules identified on transcriptome dissection in stomach carcinogenesis.

tinal tract.[54] There are reports that *CDH17* expression is correlated with lymph node metastasis of gastric cancer.[74,75] A validation study of gastric cancer on quantitative RT-PCR and immunostaining confirmed that the expression of cadherin-17 was associated with depth of tumor invasion and a poor prognosis for the patient.[71] Furthermore, significant association was observed between cadherin-17 expression and CDX2 expression as well as MUC2-positive I type gastric cancer. We have recently found that EGF induced cadherin-17 expression in gastric cancer cells. Therefore, cadherin-17 may participate in the development and progression of gastric cancers of an intestinal phenotype, partly through the EGFR pathway.

Novel genes and stomach carcinogenesis

The role of genes and molecules identified on transcriptome dissection introduced here in stomach carcinogenesis is summarized in Fig. 12. Increased expression of Reg IV and HOXA10, and reduced expression of claudin-18 may participate in the development and differentiation of gastric cancer with an intestinal phenotype. Reg IV may be involved in late stages of carcinogenesis. In contrast, GW112 may play a role in the development and progression of histologically well-differentiated gastric cancer independently from Reg IV. SPC18 may participate in tumor growth and invasion regardless of histological type and mucin phenotype. Importantly, all
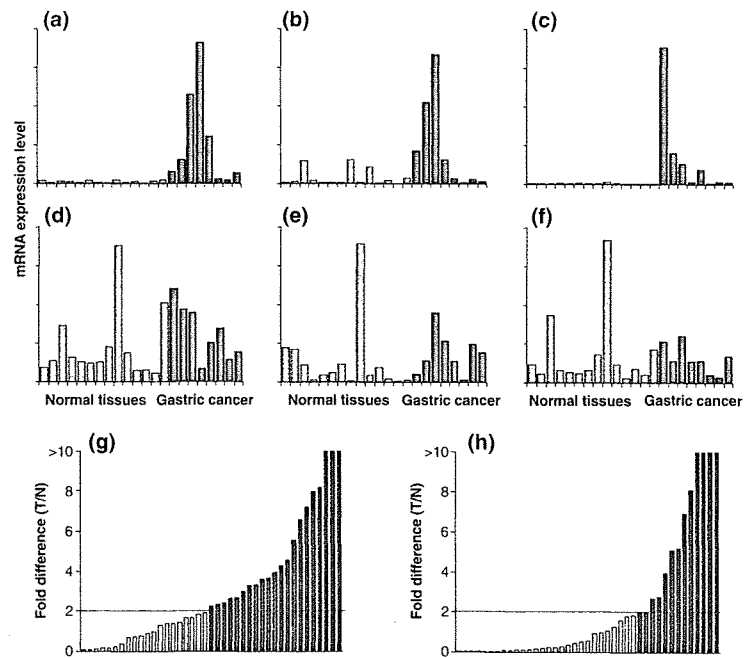
Figure 13 Representative results of quantitative reverse transcription–polymerase chain reaction of various normal tissues (brain, spinal cord, heart, skeletal muscle, lung, stomach, small intestine, colon, liver, pancreas, kidney, bone marrow, spleen, leukocytes) and gastric cancers for genes extracted by the *Escherichia coli* ampicillin trap (CAST) method. (a) Gene A; (b) gene B; (c) gene C; (d) gene D; (e) gene E; (f) gene F. (g,h) Ratio of each mRNA in gastric cancer (T) to that in corresponding non-neoplastic mucosa (N), (fold difference): (g) gene A: T/N > 2, 21/41 (51%); (h) gene C: T/N > 2, 13/41 (32%).

the genes and molecules identified on transcriptome dissection could be novel diagnostic and/or therapeutic targets.

NEW APPROACHES FOR IDENTIFYING MEMBRANE-BOUND OR SECRETED PROTEINS AS DIAGNOSTIC AND THERAPEUTIC TARGETS

Cell surface and secreted proteins are important both in basic science and clinical applications. These proteins, when overexpressed in cancer, are potential drug targets and tumor markers. We have been searching for such molecules using a novel signal sequence trap method called the *Escherichia coli* ampicillin trap (CAST) method, which was developed by Ferguson et al.[76] The principle is as follows: the mechanisms for protein translocation across prokaryotic and eukaryotic membranes are relatively conserved, and mammalian signal sequences can functionally replace those of prokaryotic genes. pCAST is a plasmid with a mutant *β-lactamase* lacking the endogenous signal peptide. A *Bam*HI site is placed upstream of, and in-frame with, the mutant *β-lactamase*, and an *Eco*RI site is included for directional cloning. When transformed with pCAST, bacteria do not grow on ampicillin-supplemented media. Survival on ampicillin is observed only when various cDNA fragments encoding a signal sequence have been inserted into this site in pCAST.

Random-primed cDNA libraries were generated from gastric cancer cell lines and normal gastric mucosal tissue, ligated into pCAST (kindly provided by Dr Jonathan Graff, University of Texas Southwestern Medical Center at Dallas),

and >1000 of the randomly selected ampicillin-resistant clones were sequenced. These included many genes encoding secreted and transmembrane proteins. By comparing the list of identified genes from gastric cancer cell lines with normal gastric mucosa, candidates overexpressed in cancer were selected. The expression was then validated on quantitative RT-PCR in gastric cancer tissues and corresponding non-neoplastic gastric mucosa in the same way as the identification through SAGE data analysis (Fig. 13). It was found that several genes for secretory and membrane proteins were definitely overexpressed in gastric cancer, and should thus be studied for their function in protein expression and cancer. From these, it is expected that further novel diagnostic and therapeutic targets will be identified.

CONCLUSIONS

Transcriptome dissection is a detailed analysis of entire expressed transcripts in affected tissues, to facilitate an understanding of the precise molecular mechanisms of pathogenesis. Transcriptome dissection of gastric cancer through SAGE-based analysis uncovered several novel genes that were specifically upregulated or downregulated in gastric cancer. These include *Reg IV, GW112, SPC18, HOXA10, and CLDN18* and so on. The information obtained from transcriptome dissection greatly contributes to development of new tools for diagnosis, treatment and prevention of cancer. In a tide of translation from the discovery of seeds to the clinical stage, we believe that our approach is in the right direction of pathology, which is located in the center of diag-

nosis and which provides a bridge between genomic research, morphological analysis and clinics.

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h-prune Is an Independent Prognostic Marker for Survival in Esophageal Squamous Cell Carcinoma

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Background: The human homologue of *Drosophila* prune (PRUNE, which encodes h-prune) protein interacts with glycogen synthase kinase 3 and promotes cell motility. The aim of our study was to investigate the impact of immunohistochemically detected h-prune expression on the survival of patients with esophageal squamous cell carcinoma (ESCC).

Methods: Immunohistochemical staining of h-prune was performed for 205 surgically resected specimens of ESCC.

Results: In total, 43 (21%) of 205 ESCC cases were positive for h-prune. h-prune-positive ESCC cases showed a more-advanced T stage ($P < 0.0001$), N stage ($P < 0.0001$), and tumor stage ($P < 0.0001$) than h-prune-negative ESCC cases. In the group of 116 stage II and III ESCC cases, recurrence of ESCC was frequently found in h-prune-positive cases. In patients with lung recurrence, the tumors were more likely to be h-prune positive than h-prune negative. Univariate analysis revealed that T stage ($P < 0.0001$), N stage ($P < 0.0001$), tumor stage ($P < 0.0001$), and h-prune staining ($P < 0.0001$) were significant prognostic factors for survival. Multivariate analysis indicated that N stage ($P = 0.0182$) and h-prune staining ($P < 0.0001$) were independent predictors for survival.

Conclusions: These results indicate that immunostaining of h-prune is useful to identify patients at high risk for recurrence or poor prognosis associated with ESCC.

Key Words: h-prune—Esophageal squamous cell carcinoma (ESCC)—Motility—Metastasis—Prognosis.

Esophageal cancer is the eighth most common cancer and the sixth leading cause of cancer-related death worldwide.[1] The two predominant forms of esophageal cancer, which differ both epidemiologically and pathologically, are squamous cell carcinoma and adenocarcinoma. Globally, squamous cell carcinoma accounts for more than 90% of esophageal

cancers.[2] Most esophageal squamous cell carcinoma (ESCC) is diagnosed at an advanced stage, and even superficial ESCC that appears to extend no further than the submucosa metastasizes to the lymph nodes in 50% of cases.[3] For localized ESCC, surgery is the primary therapeutic option. However, the prognosis is unsatisfactory, even in curatively resected patients where the five-year survival rate is <50% after surgery.[4] Several prognostic markers, such as nodal status and tumor stage, are currently accepted for clinical use. We reported previously that expression of VEGF-C and cyclin B1 are independent prognostic

factors for ESCC;[5,6] however, these markers cannot completely identify which patients are at low or high risk for disease recurrence. Therefore, identification of better prognostic markers for patients with ESCC is important.

We previously reported interaction of the human homologue of *Drosophila* prune (PRUNE, which encodes h-prune) protein with a glycogen synthase kinase 3 (GSK-3)[7]. GSK-3 inhibitors or small interfering RNAs (siRNAs) for GSK-3 and h-prune inhibit cell motility.[7] h-prune is localized to focal adhesions, and the siRNA for GSK-3 or h-prune delays the disassembly of paxillin. Tyrosine phosphorylation of focal adhesion kinase and activation of Rac are suppressed in GSK-3 or h-prune knockdown cells.[7] These results suggest that GSK-3 and h-prune act cooperatively to regulate cell motility. In contrast, h-prune has phosphodiesterase (PDE) activity with greater affinity for cAMP than cGMP as substrate.[8] Phosphodiesterases are a diverse superfamily of molecules that catalyze the hydrolysis of 3′,5′-cyclic nucleotides to their corresponding nucleoside 5′-monophosphates.[9] It has been reported that h-prune PDE activity is also involved in cell motility.[8] Because metastasis involves movement of cells from one site to another, these results suggest that overexpression of h-prune contributes to the malignant behavior of human cancers, possibly by promoting cancer cell motility.

Immunohistochemical analysis of h-prune has been reported for several human cancers. h-prune is overexpressed in breast cancers and is associated with high metastatic potential.[8] In our previous study, overexpression of h-prune correlated with T stage (depth of invasion), N stage (degree of lymph node metastasis), and tumor stage in colorectal cancers.[7] In addition, h-prune expression is an independent predictor of survival of patients with gastric cancer.[10] These data support the idea that h-prune protein has potential as a novel prognostic factor for a wide variety of malignancies. The aim of our present study was to investigate the impact of immunohistochemically detected h-prune expression on survival of patients with ESCC.

MATERIALS AND METHODS

Tissue Samples

In a retrospective study design, 205 primary tumors were collected from patients diagnosed with ESCC who underwent surgery between 1990 and 2002 at Oita University Hospital (Oita, Japan). All patients underwent curative resection. Only patients without preoperative radio- or chemotherapy and without clinical evidence of distant metastasis were enrolled in the study. Patients consisted of 182 men and 23 women. The mean age was 65 years (range, 41–84 years). Postoperative follow-up was scheduled every one, two, or three months during the first two years after surgery and every six months thereafter unless more frequent follow-up was deemed necessary. Chest X-ray, chest computed tomography (CT) scan, and serum chemistries were done at every follow-up visit. Recurrence was evaluated from records at Oita University and other hospitals. Recurrence patterns were classified into two categories, locoregional and distant.[11] Simultaneous locoregional and distant recurrence was considered as part of the distant recurrence group. For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues. Histological classification was based on the World Health Organization system. Tumor staging was performed according to the TNM stage grouping system.[12] Because written informed consent was not obtained, for strict privacy protection, identifying information for all samples was removed before analysis; this procedure is in accordance with the ethical guidelines for human genome/gene research enacted by the Japanese government.

Immunohistochemistry

One or two representative tumor blocks, including the tumor center, invading front, and tumor-associated non-neoplastic mucosa, was examined from each patient by immunohistochemistry. In cases of large, late-stage tumors, different two sections were examined to include representative areas of the tumor center as well as of the lateral and deep tumor invasive front. h-prune was detected immunohistochemically with a polyclonal antibody raised in our laboratory.[7] The specificity of the anti-h-prune antibody has been characterized in detail.[7] A Dako LSAB Kit (Dako, Carpinteria, CA, USA) was used for immunohistochemical analysis as described previously.[7] In brief, sections were pretreated with microwaving in citrate buffer for 30 min to retrieve antigenicity. After peroxidase activity was blocked with 3% H_2O_2-methanol for 10 min, sections were incubated with normal goat serum (Dako) for 20 min to block nonspecific antibody binding sites. Sections were incubated with rabbit polyclonal anti-h-prune (diluted 1:50) for one hour at room temperature. Sections were then incubated with biotinylated anti-
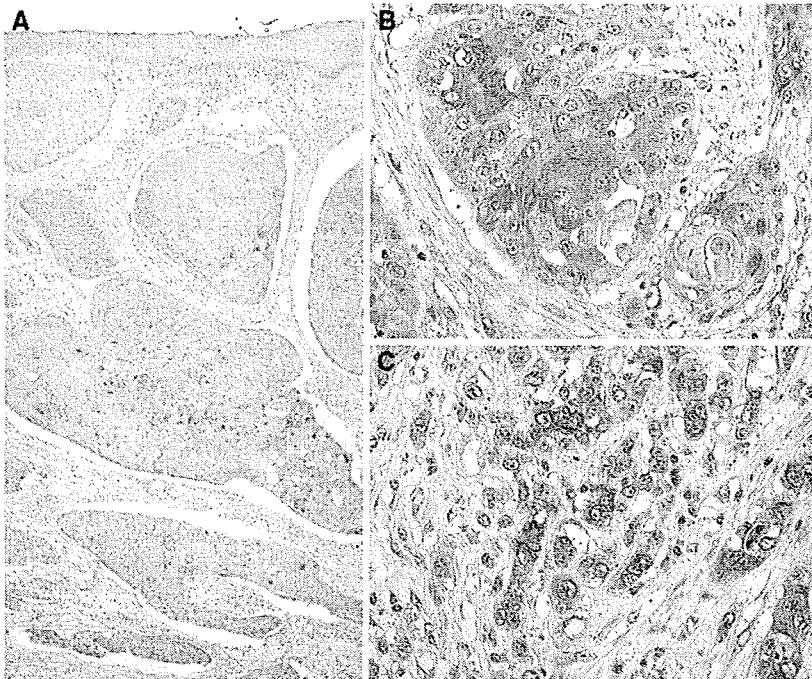
FIG. 1. Immunohistochemical analysis of h-prune in ESCC tissues. (A) In the non-neoplastic esophageal mucosa adjacent to the tumor, only weak or no staining of h-prune was observed in epithelial and stromal cells. In ESCC tissue, in addition to weak staining, strong staining was observed in the cytoplasm of some tumor cells (original magnification, × 40). (B, C) In well-differentiated (B) and poorly differentiated (C) squamous cell carcinoma, strong cytoplasmic staining of h-prune was observed in some tumor cells. Some tumor cells and stromal cells showed only weak staining (original magnification, × 400).

rabbit IgG and peroxidase-labeled streptavidin for 10 min each. Staining was completed with a 10-min incubation with the substrate-chromogen solution. The sections were counterstained with 0.1% hematoxylin. Negative controls were created by omission of the primary antibody. Expression of h-prune was scored in all tumors as positive or negative. When more than 50% of tumor cells were strongly stained, the immunostaining was considered positive for h-prune. Using these definitions, two surgical pathologists (NO and KS), without knowledge of the clinical and pathologic parameters or the patients' outcomes, independently reviewed immunoreactivity in each specimen. Interobserver differences were resolved by consensus review at a double-headed microscope after independent review.

Statistical Methods

Correlations between clinicopathological parameters and h-prune expression were analyzed by chi-square test. Kaplan–Meier survival curves were constructed for h-prune-positive and h-prune-negative patients. Survival rates were compared between h-prune-positive and h-prune-negative groups. Differences between survival curves were tested for statistical significance by log-rank test.[13] Cox proportional hazards multivariate model was used to examine the association of clinical and pathologic factors and the

expression of h-prune with survival. A P value of less than 0.05 was considered statistically significant.

RESULTS

Immunohistochemical Analysis of h-prune in Esophageal Cancer

Staining of h-prune was detected in 185 (84%) of the 205 investigated specimens of ESCC. Of the 185 ESCC cases in which h-prune staining was detected, 127 cases showed only weak staining of the cytoplasm of tumor cells. However, in 58 ESCC cases, strong staining was also observed in the cytoplasm of a subset of tumor cells (Fig. 1A). In the non-neoplastic esophageal mucosa adjacent to the tumor, only weak or no staining of h-prune was observed in epithelial and stromal cells (Fig. 1A). Strong h-prune staining was observed in both well-differentiated (Fig. 1B) and poorly differentiated ESCC (Fig. 1C). In the majority of ESCC cases containing strongly stained tumor cells, more than 50% of the tumor cells showed cytoplasmic staining for h-prune. When more than 50% of tumor cells were strongly stained, the immunostaining was considered positive for h-prune. In total, 43 (21%) of 205 ESCC cases were positive for h-prune. We analyzed the relation between h-prune expression and clinicopathologic characteristics. h-

TABLE 1. *Association of h-prune staining with clinico-pathological features of ESCC*

	h-prune staining (%)		
	Positive	Negative	P*
Age (years)			
> 60	28 (20%)	114	0.6327
≤ 61	15 (24%)	48	
Sex			
Male	40 (22%)	142	0.4716
Female	3 (13%)	20	
T stage			
Tis	0 (0%)	3	< 0.0001
T1	5 (5%)	104	
T2	7 (33%)	14	
T3	29 (41%)	41	
T4	2 (100%)	0	
N stage			
N0	9 (8%)	101	< 0.0001
N1	34 (36%)	61	
Stage			
Stage 0	0 (0%)	3	< 0.0001
Stage I	2 (2%)	84	
Stage II	15 (25%)	44	
Stage III	26 (46%)	31	
Histological differentiation[§]			
Well	13 (32%)	28	0.2711
Moderately	19 (17%)	93	
Poorly	11 (21%)	41	

* Chi-square test.

prune-positive ESCC cases showed more advanced T stage (P < 0.0001, chi-square test), N stage (P < 0.0001, chi-square test), and tumor stage (P < 0.0001, chi-square test) than h-prune-negative ESCC cases (Table 1).

Relation between H-prune Staining and Recurrence

We analyzed the relation between h-prune staining and recurrence of ESCC after curative resection. Recurrence occurred in 55 (27%) of 205 patients with ESCC. A significant relation was also found between recurrence and tumor stage (P < 0.0001). Recurrence occurred in 0% of patients with stage 0, 2% of those with stage I, 34% of those with stage II, and 58% of those with stage III. Because disease recurrence was a rare event in stage 0 and stage I ESCC cases, the relation of h-prune expression with recurrence of ESCC was investigated for stage II and stage III ESCC cases (n = 116). Of 53 ESCC cases with recurrence, 28 (53%) cases were positive for h-prune, whereas of 63 ESCC cases without recurrence, 13 (21%) cases were positive for h-prune (P = 0.0006, chi-square test). We next asked how accurate h-prune staining is for prediction of site of recurrence. As shown in Table 2, h-prune staining predicted lung recurrence with 70% accuracy.

TABLE 2. *Accuracy of h-prune staining for prediction of recurrence in stage II and III ESCC*

	h-prune staining		
	Positive	Negative	Accuracy
Recurrence			
Positive	28	25	67%
Negative	13	50	
Site of recurrence			
Lung			
Positive	9	3	70%
Negative	32	72	
Liver			
Positive	5	5	65%
Negative	36	70	
Lymph node			
Positive	13	17	61%
Negative	28	58	
Carcinomatous pleuritis			
Positive	3	4	64%
Negative	38	71	

* Chi-square test.

Relation between h-prune Staining and Survival of ESCC Patients

We next examined the relation between survival and h-prune staining in ESCC. Univariate analysis revealed that T stage (Tis–T1 vs. T2–4, P < 0.0001, log-rank test), N stage (N0 vs. N1, P < 0.0001, log-rank test), tumor stage (stage 0–I vs. stage II–III, P < 0.0001, log-rank test), and h-prune expression (P < 0.0001, log-rank test) (Fig. 2A, B, C, D) were significant prognostic factors for survival of patients with ESCC, whereas age, sex, and histological differentiation did not correlate with patient survival. We then used Cox proportional hazards multivariate model to examine the association of clinicopathological factors and expression of h-prune with survival. Multivariate analysis indicated that N stage and h-prune expression were independent predictors of survival in patients with ESCC (Table 3).

Patients with ESCC at stage 0 and stage I have a good rate of survival, whereas patients with ESCC at stage IV show a poor rate of survival. However, it is difficult to predict the survival of patients with stage II or stage III ESCC. Therefore, we analyzed the prognostic value of h-prune in patients with stage II (n = 59) and stage III (n = 57) ESCC. In the stage II ESCC group (n = 59), patients with h-prune-positive ESCC had a significantly worse survival rate than those with h-prune-negative ESCC (P = 0.0040, log-rank test) (Fig. 2E). Furthermore, in the stage III ESCC group (n = 57), patients with h-prune-positive ESCC had also a significantly worse survival rate
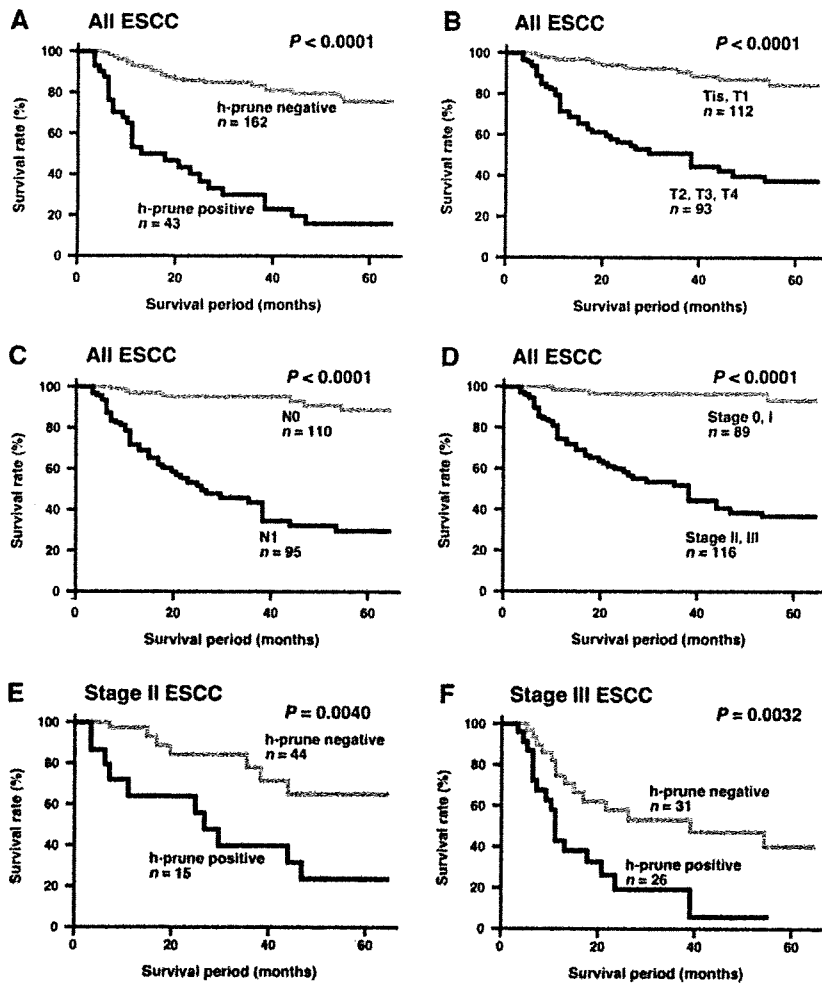
FIG. 2. Survival of patients with ESCC.
(A) Kaplan–Meier curves of patients with h-prune negative- or h-prune-positive ESCC. (B) Kaplan–Meier curves of patients with low T stage (Tis and T1) or high T stage (T2, T3, and T4) ESCC. (C) Kaplan–Meier curves of patients with low N stage (N0) or high N stage (N1) ESCC. (D) Kaplan–Meier curves of patients with early stage (stages 0 and I) or late stage (stages II and III) ESCC. (E) Kaplan–Meier curves of stage II ESCC patients with h-prune-negative and h-prune-positive tumors. (F) Kaplan–Meier curves of stage III ESCC patients with h-prune-negative and h-prune-positive tumors.

than h-prune-negative ESCC ($P = 0.0032$, log-rank test) (Fig. 2F).

DISCUSSION

The long-term survival of patients with ESCC remains poor because of the high incidence of lymph node metastasis and because of early recurrence after curative surgical resection. Therefore, it is critical to identify patients at high risk of recurrence or with poor prognosis to develop effective treatments after surgery. Overexpression of h-prune is associated with high metastatic potential and disease progression in breast, colorectal, pancreatic, and gastric cancers,[7,8,10] suggesting that h-prune protein may be a novel prognostic factor for a wide variety of malignancies. In the present study, we analyzed h-prune expression in ESCC immunohistochemically and found that h-prune-positive ESCC cases showed ad-

vanced T stage, N stage, and tumor stage. In addition, the incidence of disease recurrence was high in h-prune-positive ESCC cases. Univariate and multivariate analyses revealed that staining of h-prune is a prognostic indicator. The sensitivity and specificity for prediction of recurrence were 53% (28/53) and 79% (50/63), respectively. h-prune staining predicted recurrence with 67% accuracy.

In cases of distant metastasis, the most commonly involved organs are the lungs, bones, and liver, followed by the adrenal glands, brain, kidneys, skin, and eyes.[11] In the present study, h-prune-positive cases showed a high frequency of recurrence in the lung, indicating that during postoperative follow-up, particular attention should be paid to lung in h-prune-positive ESCC patients. In the detection of recurrence, the selected imaging modalities are important in many regards. Although both magnetic resonance imaging (MRI) and CT are useful diagnostic tools in recurrent ESCC, CT is superior to MRI in detecting

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TABLE 3. *Multivariate analysis of factors that influence survival*

Factor	Hazard ratio	(95% CI*)	χ^2	P†
T stage				
Tis/1	1	(Reference)	0.702	0.4021
T2/3/4	1.41	(0.63–3.18)		
N stage				
N0	1	(Reference)	8.182	0.0182
N1	4.57	(1.61–12.96)		
Stage				
Stage 0/I	1	(Reference)	1.004	0.3163
Stage II/III	2.41	(0.43–13.53)		
h-prune expression				
Negative	1	(Reference)	18.61	<0.0001
Positive	3.49	(1.98–6.17)		

* Confidence interval.
† Cox proportional hazards model.

lung metastasis.[14] Immunostaining of h-prune in surgically resected specimens may be useful to predict lung recurrence.

In the present study, staining of h-prune correlated with short survival rate in stage II and stage III ESCC cases. Patients diagnosed with stage II or III ESCC have variable prognoses, and they are the group that would benefit most from discovery of a prognostic factor that can identify individuals for whom adjuvant treatment would be most advantageous. To clarify whether h-prune immunostaining is useful for identification of patients most likely to benefit from adjuvant treatment, association between h-prune staining and response to adjuvant therapies should be investigated in the near future. Previous data indicated that dipyridamole, an anti-platelet aggregation drug, inhibits h-prune PDE activity and cell motility in a breast cancer cell line.[8] In the SW480 colorectal cancer cell line, knock-down of h-prune decreased cell motility.[7] These results suggest that inhibition of h-prune may be useful for preventing or treating metastasis.

The reason why h-prune-positive ESCC showed a high incidence of lung recurrence and short survival rate remains unclear. Because forced expression of h-prune promotes cell motility,[8] overexpression of h-prune contributes to the malignant behavior of ESCC, possibly by promoting cancer cell motility. h-prune also interacts with nm23-H1 (NME1, which encodes nm23-H1),[15] a known suppressor of cancer metastasis.[16] The h-prune and nm23-H1 proteins partially colocalize in the cytoplasm.[15] It has been suggested that overexpression of h-prune inhibits the anti-metastasis function of nm23-H1 during the metastatic process.[8] In ESCC, reduced expression of nm23-H1 is associated with poor prognosis of ESCC patients,[17] suggesting that inhibition of the anti-metastasis function of nm23-H1 by overexpression of

h-prune may be one reason why h-prune-positive ESCC showed a high incidence of recurrence and short survival rate.

In conclusion, we found that staining of h-prune correlates with tumor progression and reduced survival rate in patients with ESCC. Immunostaining of h-prune on surgically resected specimens may be useful for prediction of lung recurrence.

ACKNOWLEDGMENTS

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Laminin γ2 Mediates Wnt5a-Induced Invasion of Gastric Cancer Cells

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BACKGROUND & AIMS: Wnt5a expression stimulates in vitro migration and invasion of cultured gastric cancer cells by an unknown mechanism and is also correlated with aggressiveness of gastric tumors. The aim of this study was to show that Wnt5a is involved in metastasis of gastric cancer cells in vivo and to explore the molecular mechanism by which Wnt5a regulates migration and invasion. **METHODS:** In an experimental liver metastasis assay, Wnt5a-knockdown gastric cancer cells were injected into the spleens of nude mice. Microarray analyses were used to compare expression patterns between mouse fibroblast L cells that stably express wild-type and a mutant form of Wnt5a to investigate Wnt5a-dependent gene expression. The expression of genes found to be regulated by Wnt5a was investigated in cultured gastric cancer cells. Immunohistochemical analyses were performed to measure levels of Wnt-regulated gene products in 153 gastric cancer samples. **RESULTS:** Knockdown of Wnt5a in gastric cancer cells reduced the number of liver metastases that formed in nude mice. Microarray analyses indicated that Wnt5a activity induced expression of the gene encoding laminin γ2, a subunit of the epithelial basement membrane protein laminin-5. Wnt5a induced the expression of laminin γ2 through the activation of protein kinase C and c-Jun-N-terminal kinase. The invasive activity of gastric cancer cells depended on laminin γ2; Wnt5a expression levels correlated with those of laminin γ2 in diffuse-scattered type gastric tumor samples from patients. **CONCLUSIONS: Wnt5a contributes to gastric cancer progression by increasing metastatic potential. Wnt5a up-regulates laminin γ2 to mediate gastric cancer cell aggressiveness.**

G astric cancer (GC) is the fourth most common malignancy in the world and is the second leading cause of death after lung cancer.[1] Advances in diagnostic tools and treatments have led to excellent long-term survival for patients with early GC.[2] However, despite improvements in diagnostic and therapeutic strategies, the prognosis of patients with advanced GC with extensive invasion and metastasis remains poor. Several discrete steps can be discerned in the biological cascades of metastasis,[3] and several molecules have been suggested to be involved in the processes of aggressiveness in GC.[4]

It has been shown that frequent up-regulation of *Wnt5a* messenger RNA (mRNA) is detected in primary GC[5] and that expression of Wnt5a protein is associated with advanced T classification (depth of invasion) and N classification (degree of lymph node metastasis) and poor prognosis.[6] Wnt5a has also been reported to be involved in the aggressiveness of melanoma, lung cancer, and breast cancer.[7,8] Wnt5a, a member of the Wnt family of proteins, is a representative ligand that activates the β-catenin–independent pathway via mobilization of intracellular Ca^{2+} and the activation of protein kinase C (PKC), resulting in the stimulation of migration of several cultured cells, including cancer cells.[7–9] Using GC cell lines, a preceding study showed that overexpression of Wnt5a activates focal adhesion kinase and that knockdown of Wnt5a reduces the turnover number of paxillin at the focal adhesion.[6] However, whether Wnt5a is involved in invasion and metastasis of cancer cells in vivo has not yet been reported, and the molecular mechanism by which Wnt5a is involved in the aggressiveness of GC remains to be clarified.

Extracellular matrix proteins, including collagens, laminins, fibronectins, and proteoglycans, not only create tissue architecture but also regulate complex cellular functions by binding to specific cell-surface receptors, integrins. Basement membranes are thin sheets of specialized extracellular matrix proteins supporting epithelial cell layers. A major component of the epithelial basement membrane, laminin-5, has been implicated in cell migration and adhesion.[10,11] Laminin-5 promotes attachment, scattering, and migration of nontumorigenic epithelial cells through the interaction with integrins α3β1, α6β1, and α6β4 in vitro. It also stimulates human tumor cells to form marked lamellipodia, leading to enhanced cell migration and invasion.[12,13] Laminin-5 consists of 3 subunits, α, β, and γ, linked by disulfide bonds to form

a cross-shaped structure. In particular, the laminin γ2 chain has been reported to be expressed at the invasive front in tumor cells such as colon, breast, pancreas, lung, and gastric cancers,[11,14,15] showing that laminin γ2 is one of the most specific invasion markers.

In this study, to further explore the previous results concerning the effects of Wnt5a on GC, Wnt5a knock-down (KD) GC cells were generated and injected into the spleens of nude mice. Here, it is shown for the first time that Wnt5a is involved in the invasion and metastasis of GC cells in vivo. In addition, gene expression changes in Wnt5a-overexpressing cells were explored. It is shown that Wnt5a induces the expression of laminin γ2 and that laminin γ2 is involved in the aggressiveness of GC with Wnt5a expression.

Materials and Methods

Animals and Implantation of Tumor Cells

After 6-week-old male Balb/cAnN Crj-nu mice (Charles River Laboratory Japan, Inc, Kanagawa, Japan) were anesthetized with pentobarbital (0.05 mg/g body wt), 2.5×10^5 KKLS cells or 1×10^6 TMK-1 cells in 50 μL Hanks' balanced salt solution were injected into the spleen through a 31-gauge needle. Five weeks later, under deep anesthesia with ether, hepatectomy and splenec-tomy were performed. The number of metastatic liver tumors was counted and tumor sizes were measured. For tumor growth assays, 1×10^6 KKLS cells in 50 μL Hanks' balanced salt solution were injected into the subserosa of the stomach orthotopically. Four weeks later, under deep anesthesia with ether, gastrectomy was performed and tumor weight was measured. The protocols used for all animal experiments in this study were approved by the Animal Research Committee of Hiroshima University.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) assay was performed using a OneDay ChIP Kit (Diagnode, Philadelphia, PA) in accordance with the manufacturer's instructions. Cells were cross-linked with formaldehyde, lysed, and sonicated to shear DNA to small sizes. Sheared chromatin samples were incubated with anti-JunD or anti-c-jun antibody. The DNA fragments were purified from the immunocomplexes and used as a template for polymerase chain reaction (PCR). The PCR primers were designed for the fragment containing activator protein-1 (AP-1) sites of the promoter region of *LAMC2* (*laminin γ2* gene). The detailed method of ChIP assay was described in the Supplementary information (see Supplemental Material).

Tissue Samples

For immunohistochemical analysis, archival for-malin-fixed, paraffin-embedded tissues from 153 patients who had undergone surgical excision for GC were used. Histologic classification was made according to the Lau-ren classification system.[16] In addition, diffuse-type GCs were further classified into diffuse-adherent and diffuse-scattered subtypes.[6] Tumor staging was according to the TNM staging system. The procedure to protect the pri-vacy of patients was in accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese government.

Immunohistochemistry

The samples were sectioned, deparaffinized, and stained with H&E to ensure that the sectioned block contained tumor cells. Adjacent sections were then immu-nohistochemically stained. Immunostaining for Wnt5a, β-catenin, and laminin γ2 was performed as described.[6,15] When more than 50% of cancer cells were stained, the immunostaining was considered positive. The detailed immunohistochemical analyses are described in the Sup-plementary information (see supplemental material on-line at www.gastrojournal.org).

Results

Wnt5a Is Involved in In Vivo Metastasis of GC Cells

To examine the in vivo relationship between ex-pression of Wnt5a and metastasis in cancer cells, the present study generated 2 GC cell lines stably expressing small interfering RNA (siRNA) for Wnt5a: KKLS/Wnt5a KD cells and TMK-1/Wnt5a KD cells (Supplementary Figure 1A). KKLS cells were established from a lymph node metastasis of a patient with undifferentiated GC.[17] TMK-1 cells were established from a xenotransplantable tumor (SC-6-JCK) in nude mice, which originated from the lymph node metastasized tumor of human GC.[4] These Wnt5a KD GC cells showed reduced in vitro cell migration and invasion activities in Transwell assays as compared with control cells (Supplementary Figure 1B). However, reduction of Wnt5a did not affect the cell growth of these GC cells (Supplementary Figure 1C).

KKLS/Wnt5a KD cells, TMK-1/Wnt5a KD cells, and their control cells were injected into the spleens of nude mice to observe experimental liver metastasis. Although the metastatic liver tumor sizes were similar in KKLS/control and KKLS/Wnt5a KD cells, knockdown of Wnt5a in KKLS cells significantly decreased the numbers of the experimental liver metastatic nodules (Figure 1A and B) ($P = .0060$). When the cells were implanted in gastric subserosa, primary tumors were formed and their tumor weights in the stomach were statistically similar for con-trol and Wnt5a KD cells ($P = .6098$) (Figure 1C). Similar results were observed in TMK-1 cells (Figure 1D). These are the first in vivo results showing that Wnt5a is re-quired for invasion and metastasis rather than growth in GC cells. However, because in vivo metastatic experi-
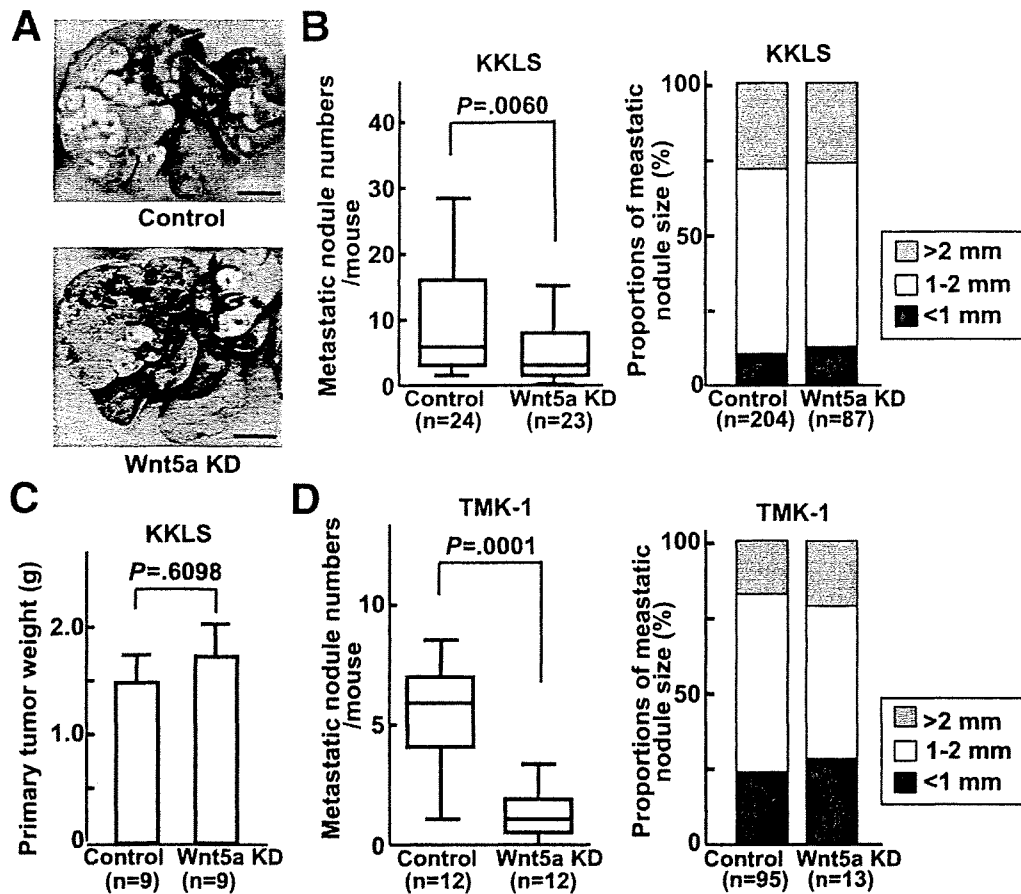
Figure 1. Wnt5a silencing reduces the metastatic potential of GC cells. (A and B) Liver tumor colonies were produced by the intrasplenic injection of KKLS cells. Photographs representing (A) liver metastases, (B, *left panel*) numbers of metastatic nodules per mouse, and (B, *right panel*) apparent sizes of each metastatic nodule are shown. *Scale bars* = 5 mm. n = 24 and n = 23 indicate the numbers of mice into which KKLS/control and KKLS/Wnt5a KD cells, respectively, were injected. n = 204 and n = 87 indicate the total numbers of metastatic nodules produced by KKLS/control and KKLS/Wnt5a KD cells, respectively. The statistical comparison was performed using Mann–Whitney U test. (C) Orthotropic implantation was performed by the injection of KKLS cells in the stomach. n = 9 indicates the number of mice into which KKLS/control or KKLS/Wnt5a KD cells were injected. The statistical comparison was performed using unpaired Student t test. (D) The numbers and sizes of liver tumor colonies produced by the intrasplenic injection of TMK-1 cells were measured. n = 12 indicates the number of mice into which TMK-1/control or TMK-1/Wnt5a KD cells were injected. n = 95 and n = 13 indicate the total numbers of metastatic liver nodules produced by TMK-1/control and TMK-1/Wnt5a KD cells, respectively. The statistical comparison was performed using Mann–Whitney U test.

ments with nude mice also reflect the ability of tumor cells to proliferate in a different organ (hepatic) microenvironment,[18] it is still possible that Wnt5a affects GC cell growth in a distant site.

Wnt5a Drives Changes in Gene Expression

Next it was attempted to analyze the mechanisms of aggressiveness of GC, commencing with a broad survey of the gene expression patterns affected by the expression of Wnt5a. To this end, mouse fibroblast L cells stably expressing Wnt5a or Wnt5a CA were used.[9] Wnt5a CA is a mutant that is not posttranslationally palmitoylated at Cys104 and loses its biological activity. Candidate gene lists were created by determining which molecules were overexpressed by ≥1.5-fold in L/Wnt5a cells but did not change in L/Wnt5a CA cells as compared with control cells (Supplementary Table 1). Laminin γ2 was further investigated among the candidate genes sug-

gested to be involved in migration and invasion, because it was reported that laminin γ2 can be detected at the invasive front of various cancers.[11,14,15]

Laminin γ2 is a subunit of laminin-5, which is found in the epithelial basement membrane.[10] mRNA levels of Wnt5a and LAMC2 were analyzed in 4 different GC cell lines: MKN-1, TMK-1, MKN-45, and KKLS cells. MKN-1 and MKN-45 cells were established from metastatic foci to lymph nodes and liver, respectively, of human GC.[4] mRNA levels of Wnt5a and LAMC2 were variable, depending on the cell type. Although MKN-1, TMK-1, and MKN-45 cells showed expression of LAMC2 mRNA, KKLS cells barely produced LAMC2 mRNA (Figure 2A). The expression of Wnt5a mRNA in MKN-45 cells was lower than that of other cells (Figure 2A). TMK-1/Wnt5a KD cells reduced the mRNA level of LAMC2 as compared with control cells (Figure 2B). When purified