noma; MKN-28; MKN-74, well-differentiated adenocarcinoma; and MKN-45, poorly differentiated adenocarcinoma) were kindly provided by Dr. Toshimitsu Suzuki. All cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (BioWhittaker, Walkersville, MD) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. For constitutive expression of olfactomedin 4, cDNA was amplified by PCR and subcloned into pcDNA 3.1 (Invitrogen, Carlsbad, CA). Transient transfection was carried out with the FuGENE6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN).

Production of olfactomedin 4 monoclonal antibodies

BALB/c mice were i.m. injected with pcDNA3.1-olfactomedin 4 into the anterior tibial muscle and pulsed with an electric pulse generator (CUY-21, BEX, Tokyo, Japan) using a 1.0 cm-diameter round plate electrode twice with a 2 week interval between injections. Fused spleen cells with NS-1 were cultured in HY soft agar with hypoxanthine-aminopterin-thymidine medium. Visible hybridoma colonies were selected and screened for production of olfactomedin 4 mAb by ELISA. We generated 2 monoclonal antibodies against olfactomedin 4 which were designated as N212 and U21-2.

Western blot analysis

For Western blot analysis, cells were lysed as described previously.[13] The culture media were concentrated with the PROTEIN Concentrate Kit (Takara Bio, Shiga, Japan). The lysates (40 μg) were solubilized in Laemmli sample buffer by boiling and then subjected to 10% SDS-polyacrylamide gel electrophoresis followed by electrotransfer onto a nitrocellulose filter. Peroxidase-conjugated anti-mouse IgG was used in the secondary reaction. Immunocomplexes were visualized with an ECL Western Blot Detection System (Amersham Biosciences, Piscataway, NJ). β-actin (Sigma, St. Louis, MO) was also stained as a loading control.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA), and 1 μg of total RNA was converted to cDNA with a First Strand cDNA Synthesis Kit (Amersham Biosciences). Quantitation of *OLFM4* mRNA levels was done by real-time fluorescence detection as described previously.[14] *OLFM4* primer sequences were 5′- TGG TGA ACA TCA GCA AAC CG -3′ and 5′- TCC CTA CCC CAA GCA CCA TA -3′. PCR was performed with a SYBR Green PCR Core Reagents Kit (Applied Biosystems, Foster City, CA). Real-time detection of the emission intensity of SYBR green bound to double-stranded DNA was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as described previously.[15] *ACTB*-specific PCR products were amplified from the same RNA samples and served as an internal control.

Tissue samples

In all, 167 primary tumors (73 women and 94 men; age range, 35–88 years; mean, 69 years) and 123 serum samples (54 women and 69 men; age range, 35–88 years; mean, 69 years) were collected from patients diagnosed with GC. 50 serum samples (28 women and 22 men; age range, 41–88 years; mean, 69 years) were also collected from patients diagnosed with CRC. Patients were treated at the Hiroshima University Hospital or an affiliated hospital.

For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from 167 patients who had undergone surgical excision of GC. Of the 167 patients, 58 had early GC and 109 had advanced GC. Early GC is limited to the mucosa or the mucosa and submucosa regardless of nodal status. Advanced GC is a tumor that has invaded beyond the muscularis propria.[16] Information on patient survival was available for 73 of the 109 advanced GC cases. Among 167 GC cases used for immunohistochemical analysis, serum samples were available for ELISA from 59 GC cases. In addition, serum samples from 64 patients with GC were analyzed by ELISA. These 64 primary GC tissue samples were not available because of lack of tumor tissue samples. In total, the serum samples from 123 patients with GC were analyzed by ELISA. Serum samples were collected before surgery and before initiation of therapy, and were stored at −80°C until analysis. Serum samples from 20 patients with chronic-active gastritis with *Helicobacter pylori* infection (7 women and 13 men; age range, 57–85 years; mean, 69 years) were also collected. Control serum samples were obtained from 76 healthy individuals (26 women and 50 men; age range, 32–79 years; mean, 60 years).

Tumor staging was according to the TNM classification system.[17] Histological classification of GC was carried out according to the Lauren classification system.[18] Because written informed consent was not obtained, for strict privacy protection, identifying information for all samples was removed before analysis. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government.

Immunohistochemistry

Formalin-fixed and paraffin-embedded samples were sectioned, deparaffinized, and stained with H&E to ensure that the sectioned block contained tumor cells. Adjacent sections were then stained immunohistochemically with a Dako Envision+ Mouse Peroxidase Detection System (Dako Cytomation, Carpinteria, CA). Antigen retrieval was done by microwave heating in citrate buffer (pH 6.0) for 30 min. After peroxidase activity was blocked with 3% H_2O_2-methanol for 10 min, sections were incubated with normal goat serum (Dako Cytomation) for 20 min to block nonspecific antibody binding sites. Sections were incubated with primary antibodies against olfactomedin 4 (1:50) for 1 hr at room temperature, followed by incubations with Envision+ anti-mouse peroxidase for 1 hr. Staining was completed with 10 min incubation with the substrate-chromogen solution. Sections were counterstained with 0.1% hematoxylin. Immunostaining of Reg IV was performed as described previously.[6]

Phenotypic analysis of GC

GCs were classified into 4 phenotypes: gastric (G) type, intestinal (I) type, gastric and intestinal mixed (GI) type and unclassified (N) type. For phenotypic expression analysis of GC, we performed immunohistochemical analysis (as described above) with 4 antibodies: anti-MUC5AC (Novocastra, Newcastle, UK) as a marker of foveolar epithelial cells in the stomach, anti-MUC6 (Novocastra) as a marker of pyloric gland cells in the stomach, anti-MUC2 (Novocastra) as a marker of goblet cells in the small intestine and colorectum and anti-CD10 (Novocastra) as a marker of microvilli of absorptive cells in the small intestine and colorectum. The criteria[19] for the classification of G type and I type GCs were as follows. GCs in which more than 10% of cells in the section expressed at least 1 gastric epithelial cell marker (MUC5AC or MUC6) or intestinal epithelial cell marker (MUC2 or CD10) were classified as G type or I type cancers, respectively. Sections that showed both gastric and intestinal phenotypes were classified as GI type, and those that lacked both the gastric and intestinal phenotypes were classified as N type.

ELISA

For measurement of the serum concentration of olfactomedin 4, a sandwich ELISA was developed. First, polystyrene microtiter plates were coated with mouse monoclonal anti-olfactomedin 4 antibody (N212) by overnight incubation of 50 μL/125 ng/well antibody diluted in Tris buffer (pH 7.4). The plates were then washed 3 times with washing buffer. After the plates were blocked with 1% milk in PBS, 50 μL of recombinant olfactomedin 4 standard or sample were added to each well and incubated overnight at 4°C. After three washes, 50 μL of biotinylated mouse monoclonal anti-olfactomedin 4 antibody (U21-2) in assay buffer (1% bovine

serum albumin (BSA), Tris buffer [pH 7.4], 0.05% normal goat serum) were added to each well (75 ng antibody/well). The mixture was then incubated for 1 hr with shaking at 37°C and washed 3 times with washing buffer. The plates were incubated with 50 μL/well alkaline phosphatase-conjugated streptavidin (Dako) diluted 2,000-fold in diluent containing 1% BSA and Tris buffer (pH 7.4) for 1 hr at 37°C and washed 3 times. Color development was performed with the addition of pNPP chromogenic substrate (Sigma) followed by incubation at 37°C for 1 hr. Absorbance at 405 nm was measured with an ELISA plate reader. As a reference standard, known concentrations of human recombinant olfactomedin 4 from 0 to 270 ng/mL were tested in triplicate.

Measurement of Reg IV, CA19-9 and CEA

For measurement of the serum Reg IV concentration, a sandwich ELISA method was performed as described previously.[7] In our previous study, the cutoff level for Reg IV was set at 2.00 ng/mL.[7] CA19-9 and CEA were measured with a commercially available automated immunoassay method (Modular Analytics, Roche Diagnostics). The upper limits of normal for this method are 37 U/mL for CA19-9 and 5.0 ng/mL for CEA.

Statistical methods

Associations between clinicopathologic parameters and olfactomedin 4 expression were analyzed by Fisher's exact test. Kaplan-Meier survival curves were constructed for olfactomedin 4-positive and olfactomedin 4-negative patients. Survival rates were compared between olfactomedin 4-positive and olfactomedin 4-negative groups. Differences between survival curves were tested for statistical significance by a log-rank test.[20] Differences in the serum concentration of olfactomedin 4 between the 2 groups were tested by the nonparametric Mann-Whitney *U* test. Correlation between the serum concentration of olfactomedin 4 and that of Reg IV, CEA or CA19-9 was assessed by Spearman's correlation coefficient. A p value of less than 0.05 was considered statistically significant.

Results

Western blotting

We generated 2 monoclonal antibodies against olfactomedin 4 designated as N212 and U21-2. The monoclonal anti-olfactomedin 4 antibody (N212) detected a single band of approximately 57-kDa on Western blots of both cell extracts and culture media from MKN-45 cells (Fig. 1*a*). These results are consistent with those of a previous report.[8] We confirmed that the *OLFM4* mRNA levels determined by quantitative RT-PCR were consistent with the olfactomedin 4 protein levels determined by Western blotting (Fig. 1*b*). Moreover, we performed Western blot analysis of cell

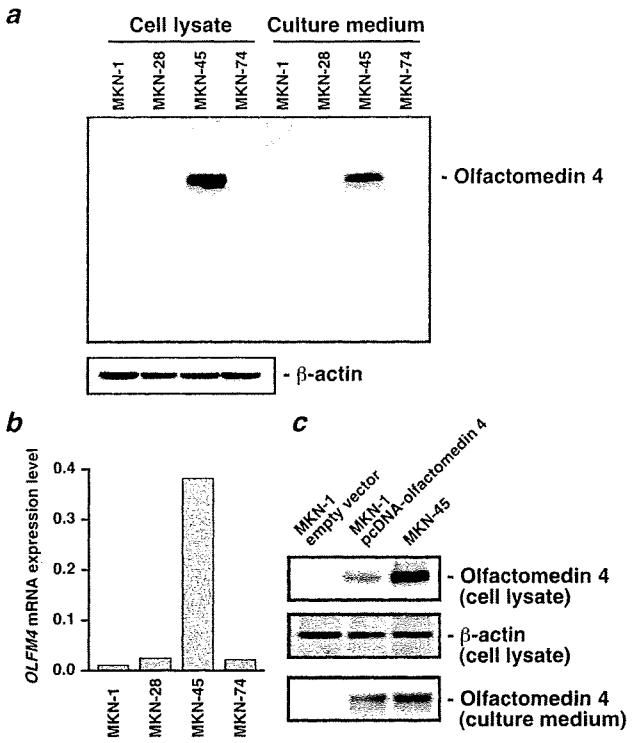

FIGURE 1 – Expression of olfactomedin 4 in GC cell lines. (*a*) Western blot analysis of olfactomedin 4 with anti-olfactomedin 4 antibody (N212). In cell lysates, an ~57 kD band is visible in MKN-45 cells. Culture medium of MKN-45 cells contained olfactomedin 4. (*b*) Quantitative RT-PCR analysis showed strong expression of *OLFM4* in MKN-45 cells, which is consistent with the Western blot results. (*c*) Western blot analysis of extracts of MKN-1 cells transiently transfected with pcDNA 3.1 (empty vector) or pcDNA-olfactomedin 4. With anti-olfactomedin 4 antibody (N212), an ~57 kD band corresponding to olfactomedin 4 is visible. MKN-45 extract served as a positive control.

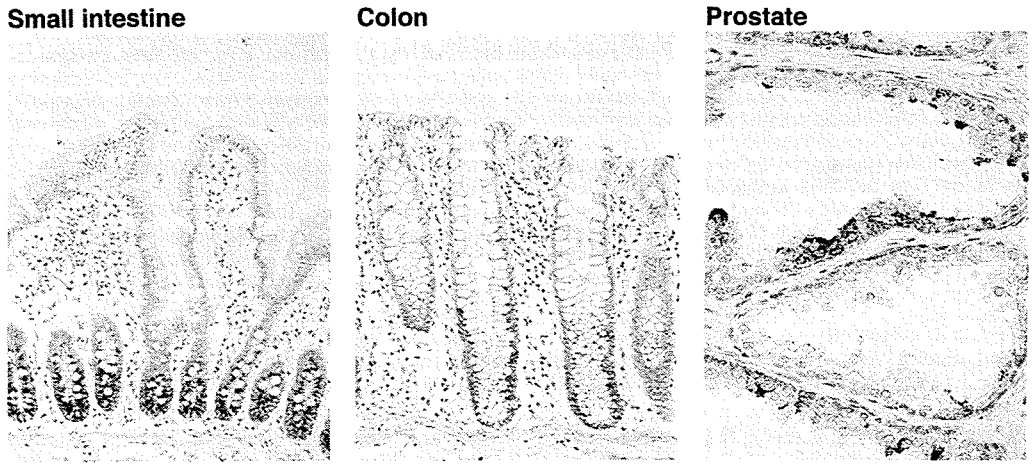

FIGURE 2 – Immunohistochemical analysis of olfactomedin 4 in non-neoplastic human tissues. In non-neoplastic small intestine, immunohistochemical analysis by N212 antibody revealed that olfactomedin 4 was expressed in the basal crypt epithelium in the small intestine. Original magnification: ×200. In non-neoplastic colon, immunohistochemical analysis by N212 antibody showed that olfactomedin 4 was expressed in the basal crypt epithelium in the colon. Original magnification: ×200. In non-neoplastic prostate, selected epithelial cells were stained by N212 antibody. Original magnification: ×400.

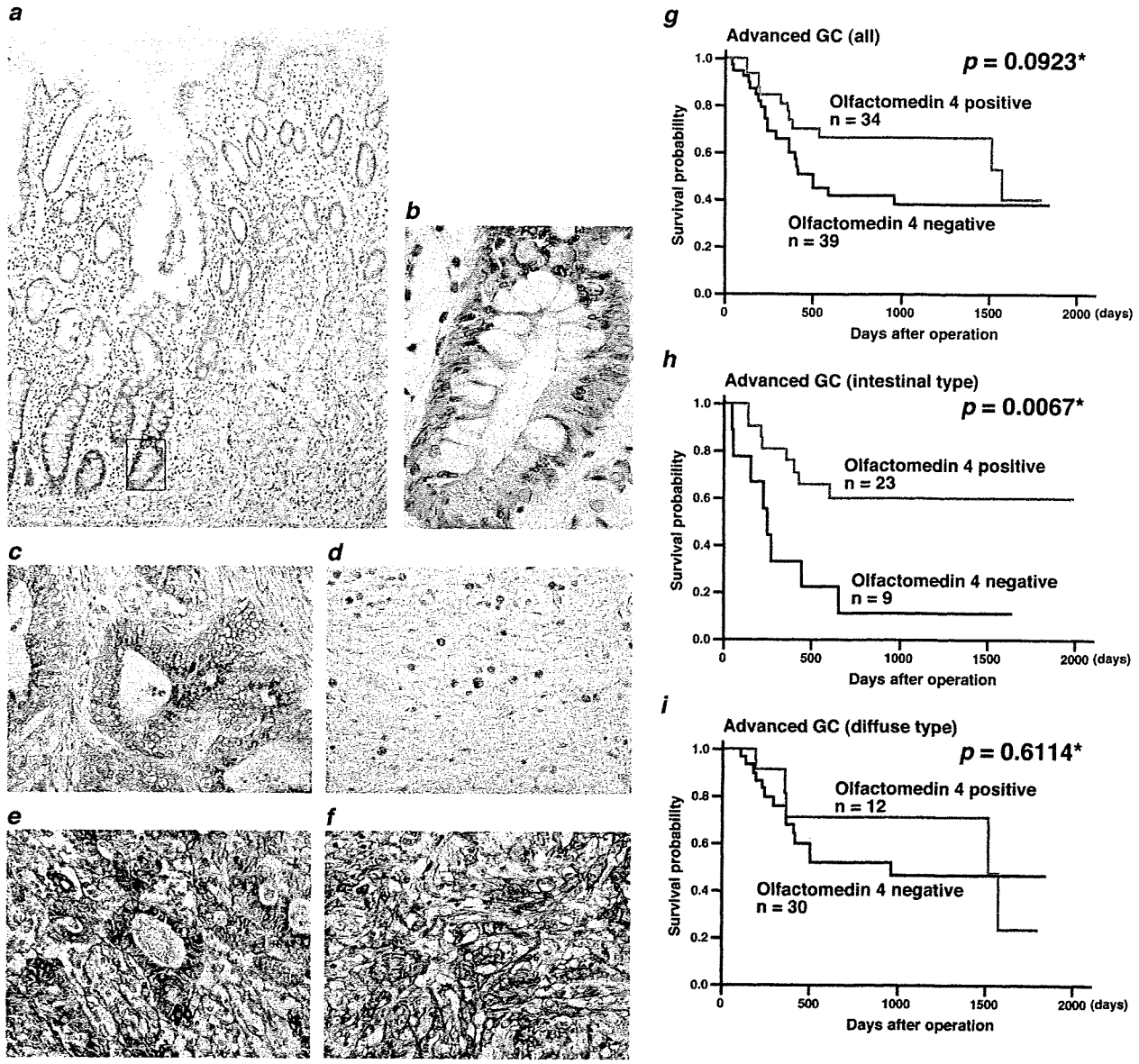

FIGURE 3 – Immunohistochemical analysis of olfactomedin 4 in GC tissues. (*a*) In non-neoplastic gastric mucosa, foveolar epithelium was not stained by N212 antibody, whereas olfactomedin 4 was expressed in the basal crypt epithelium in the intestinal metaplasia. Original magnification: ×100. (*b*) Apical granular immunoreactivity in the epithelium of intestinal metaplasia was observed. Original magnification: ×1,000. (*c*) Immunohistochemical analysis by N212 antibody in intestinal type GC. Staining of olfactomedin 4 was detected in cytoplasm of tumor cells. Original magnification: ×400. (*d*) Immunohistochemical analysis by N212 antibody in diffuse type GC. Staining of olfactomedin 4 was detected in cytoplasm of tumor cells. Original magnification: ×400. (*e*) Immunohistochemical analysis by N212 antibody in intestinal type GC. Both cytoplasmic and extracellular staining of olfactomedin 4 was detected. Original magnification: ×400. (*f*) Immunohistochemical analysis by N212 antibody in diffuse type GC. Both cytoplasmic and extracellular staining of olfactomedin 4 was detected. Original magnification: ×400. (*g–i*) Prognostic value of olfactomedin 4 staining. (*g*) In the group of 73 advanced GC patients, there was no association between olfactomedin 4 staining and survival rate. (*h*) In the group of 32 advanced intestinal type GC patients, those with olfactomedin 4-positive GC had a better survival rate than patients with olfactomedin 4-negative GC. (*i*) In the group of 41 advanced diffuse type GC patients, survival rate was not statistically different between patients with olfactomedin 4-positive GC and those with olfactomedin 4-negative GC. *log-rank test.

extracts of MKN-1 cells transiently transfected with pcDNA 3.1 or pcDNA-olfactomedin 4. We detected an ~57 kDa band corresponding to olfactomedin 4 (Fig. 1*c*). Analyses with the U21-2 antibody produced almost the same results (data not shown). These results indicate that both N212 and U21-2 antibodies recognize olfactomedin 4 protein specifically.

Immunohistochemical analysis of olfactomedin 4 in non-neoplastic small intestine, colon and prostate

Because *OLFM4* is predominantly expressed in bone marrow, small intestine, colon and prostate,[8] we performed immunohistochemical analysis of olfactomedin 4 in non-neoplastic small intestine, colon and prostate (Fig. 2). It has been reported that in small intestine, the enterocytes of the luminal surface and crypt show strong diffuse cytoplasmic immunoreactivity of olfactomedin 4.[10] Immunohistochemical analysis by N212 antibody revealed that olfactomedin 4 was expressed in the basal crypt epithelium in the small intestine; however, luminal surface epithelium was not stained. As reported previously,[10] immunohistochemical analysis by N212 antibody showed that olfactomedin 4 was expressed in the basal crypt epithelium in the colon. Specific immunostaining of small intestine or colon mucosa was not seen with preabsorbed

TABLE I – RELATIONSHIP BETWEEN OLFACTOMEDIN 4 EXPRESSION AND CLINICOPATHOLOGIC CHARACTERISTICS IN GASTRIC CANCER

	Olfactomedin 4 expression		p value[1]
	Positive	Negative	
Age			
≤65	34 (61%)	22	0.5089
>65	60 (54%)	51	
Sex			
Male	50 (53%)	44	0.4320
Female	44 (60%)	29	
T classification[2]			
T1	40 (69%)	18	0.0215
T2/3/4	54 (50%)	55	
N classification[3]			
N0	49 (61%)	31	0.2744
N1/2/3	45 (52%)	42	
Stage			
Stage I/II	64 (64%)	36	0.0172
Stage III/IV	30 (45%)	37	
Histological classification			
Intestinal	62 (72%)	24	< 0.0001
Diffuse	32 (40%)	49	
Reg IV expression			
Positive	18 (40%)	27	0.0136
Negative	76 (62%)	46	

[1]Fisher's exact test.–[2]T1, tumor invades lamina propria or submucosa; T2, tumor invades muscularis propria or subserosa; T3, tumor penetrates serosa; T4, tumor invades adjacent structures.–[3]N0, no regional lymph node metastasis; N1, metastasis in 1–6 regional lymph nodes; N2, metastasis in 7 to 15 regional lymph nodes; N3, metastasis in more than 15 regional lymph nodes.

anti-olfactomedin 4 antibody (data not shown). In prostate, selected epithelial cells were stained by anti-olfactomedin 4 antibody. Expression of olfactomedin 4 was not detected in stromal cells, such as inflammatory cells and fibroblasts. Analyses with the U21-2 antibody produced almost the same results (data not shown).

Immunohistochemical analysis of olfactomedin 4 in GC

We performed immunohistochemical analysis of olfactomedin 4 by N212 antibody in 167 GC cases. In non-neoplastic gastric mucosa, foveolar epithelium was not stained by anti-olfactomedin 4 antibody, whereas olfactomedin 4 was expressed in the basal crypt epithelium in the intestinal metaplasia (Fig. 3*a*). As reported previously,[10] apical granular immunoreactivity in the epithelium of intestinal metaplasia was observed (Fig. 3*b*). Expression of olfactomedin 4 was not detected in stromal cells, such as inflammatory cells and fibroblasts. In contrast, GC tissue showed stronger, more extensive staining than corresponding non-neoplastic mucosa. Olfactomedin 4 staining was observed in both intestinal type GC (Fig. 3*c*) and diffuse type GC (Fig. 3*d*). In general, staining of olfactomedin 4 was detected in the cytoplasm of tumor cells. The percentage of olfactomedin 4-stained tumor cells ranged from 0 to 80%. It has been reported that a loss/reduction of olfactomedin 4 expression at the front of the invasion is observed in colorectal cancer[12]; however, the tendency for loss of olfactomedin 4 expression at the invasive front was not observed. In addition to cytoplasmic staining, extracellular staining of olfactomedin 4 was observed. The immunoreactivity for the olfactomedin 4 was irregular and fibrous around tumor cells scattered in the stroma. Extracellular staining of olfactomedin 4 was detected around tumor cells showing olfactomedin 4 staining in both intestinal (Fig. 3*e*) and diffuse type GC (Fig. 3*f*). Extracellular staining of olfactomedin 4 was focal, and the percentage of olfactomedin 4-stained area in tumor-associated stroma ranged from 0 to 20%. A tendency for extracellular staining of olfactomedin 4 at the invasive front was not observed.

Next, the relationship of olfactomedin 4 staining to clinicopathologic characteristics was investigated (Table I). The level of olfactomedin 4 immunoreactivity was first evaluated in tumor cells. When more than 10% of tumor cells were stained, the immunostaining was considered positive for olfactomedin 4. In total, 94 (56%) of 167 GC cases were positive for olfactomedin 4. Olfactomedin 4 staining was observed more frequently in T1 (T1, tumor invades lamina propria or submucosa) cases (69%) than in T2/3/4 (T2, tumor invades muscularis propria or subserosa; T3, tumor penetrates serosa; T4, tumor invades adjacent structures) cases (50%, $p = 0.0215$, Fisher's exact test). Olfactomedin 4 staining was observed more frequently in stage I/II cases (64%) than in stage III/IV cases (45%, $p = 0.0172$, Fisher's exact test). Moreover, olfactomedin 4 staining was detected more frequently in intestinal type GC (72%) than in diffuse type GC (40%, $p < 0.0001$, Fisher's exact test). Olfactomedin 4 staining was not correlated with age, sex or N classification (N0, no regional lymph node metastasis; N1, metastasis in 1 to 6 regional lymph nodes; N2, metastasis in 7 to 15 regional lymph nodes; N3, metastasis in more than 15 regional lymph nodes). In the group of 73 advanced GC patients, those with olfactomedin 4-positive GC showed a tendency to have a better survival rate than patients with olfactomedin 4-negative GC; however, no statistically significant difference was found ($p = 0.0923$, log-rank test) (Fig. 3*g*).

A variety of genetic and epigenetic alterations are associated with GC; some are found in both the intestinal and diffuse types, whereas others are type-specific.[21] Because olfactomedin 4 expression was frequently found in intestinal type GC, the relationship of olfactomedin 4 expression to clinicopathologic characteristics was investigated in the group of 86 intestinal type GC cases. Expression of olfactomedin 4 was not correlated with age, sex, T classification, N classification or tumor stage (data not shown). In contrast, in the group of 32 advanced GC patients, those with olfactomedin 4-positive GC had a better survival rate than patients with olfactomedin 4-negative GC ($p = 0.0067$, log-rank test) (Fig. 3*h*). The relationship of olfactomedin 4 staining to clinicopathologic characteristics was also investigated in the group of 81 diffuse type GC cases. Olfactomedin 4 staining was observed more frequently in T1 cases (14/21, 67%) than in T2/3/4 cases (18/60, 30%, $p = 0.0044$, Fisher's exact test). Olfactomedin 4 staining was not correlated with age, sex, N classification or tumor stage (data not shown). In the group of 41 advanced GC patients, survival rate was not statistically different between patients with olfactomedin 4-positive GC and those with olfactomedin 4-negative GC ($p = 0.6114$, log-rank test) (Fig. 3*i*).

The level of olfactomedin 4 immunoreactivity was also evaluated in tumor-associated stroma. Stromal olfactomedin 4 staining was considered positive if any tumor-associated stroma was stained. In total, 54 (32%) of 167 GC cases were positive for stromal olfactomedin 4. Stromal olfactomedin 4 staining was detected more frequently in intestinal type GC (62/86, 72%) than in diffuse type GC (32/81, 40%, $p < 0.0001$, Fisher's exact test). Stromal olfactomedin 4 staining was not correlated with age, sex, T classification, N classification or tumor stage (data not shown). In the group of 73 advanced GC patients, survival rate was not statistically different between patients with stromal olfactomedin 4-positive GC and those with stromal olfactomedin 4-negative GC (data not shown).

Olfactomedin 4 is expressed in GC with gastric phenotype

We further investigated the association between olfactomedin 4 expression and the mucin phenotype because olfactomedin 4 was detected in intestinal metaplasia of the stomach, small intestine and colon. Gastric and intestinal markers were detected in 72 of 167 (43%) cases for MUC5AC, 18 (11%) cases for MUC6, 54 (32%) cases for MUC2 and 19 (11%) cases for CD10. Although olfactomedin 4 was detected in intestinal metaplasia of the stomach, small intestine and colon, olfactomedin 4 expression was frequently observed in G type GC. In G type GC, in which MUC5AC but not MUC2 was expressed, olfactomedin 4 expression was frequently detected (Figs. 4*a*–4*c*). However, all

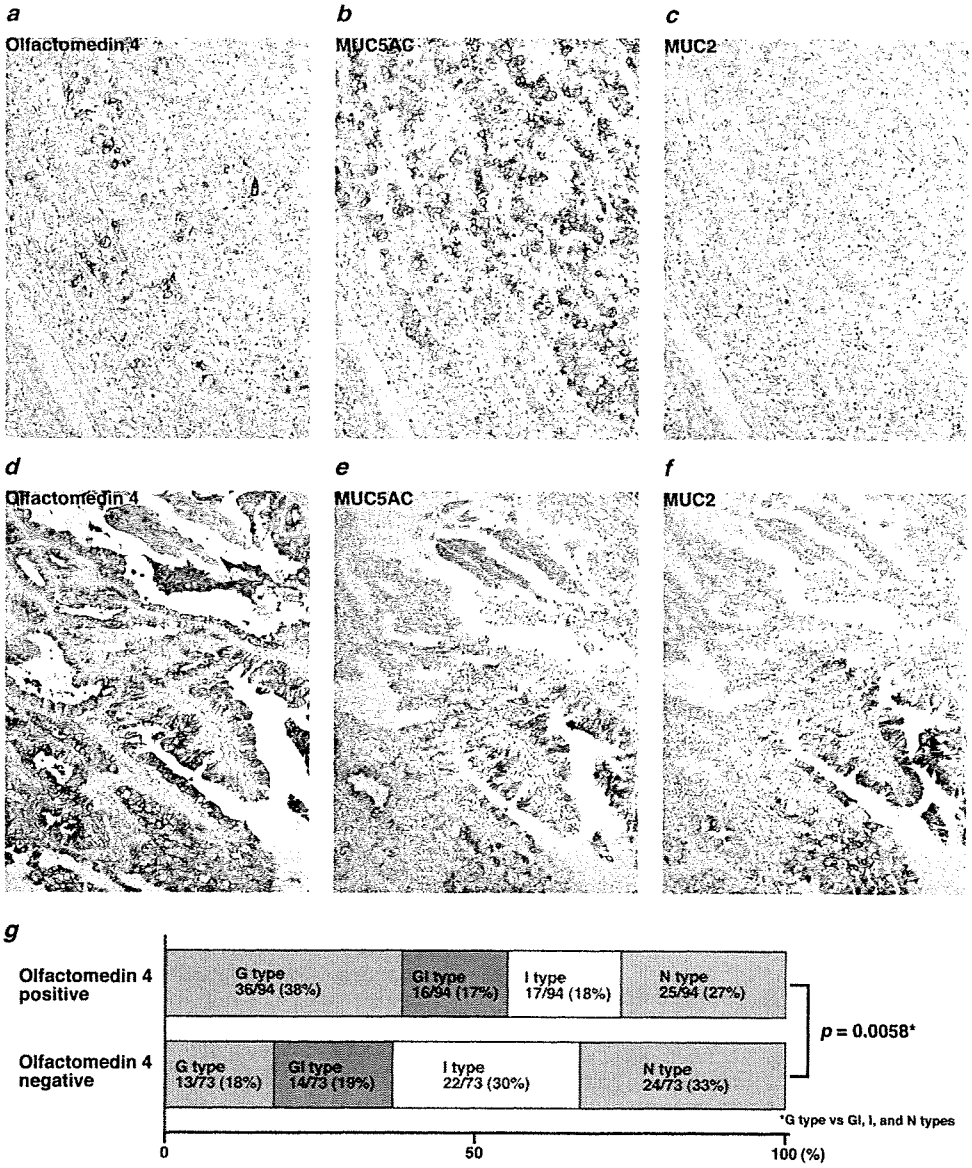

FIGURE 4 – Expression of GC phenotype. (*a–c*) G type GC. (*a*) In G type GC, olfactomedin 4 expression was frequently observed. (*b*) Expression of MUC5AC was found. (*c*) Expression of MUC2 was not found. (*d–f*) GI type GC. (*d*) In GI type GC, olfactomedin 4 expression was frequently observed. (*e*) Expression of MUC5AC was found. (*f*) Expression of MUC2 was found. (*g*) Summary of olfactomedin 4 expression and expression of GC phenotype. Expression of olfactomedin 4 occurred more frequently in G-type GC than in other (GI, I, and N) GC types. (*a–f*) Original magnification: ×100. *Fisher's exact test. G, I, GI and N indicate gastric, intestinal, gastric and intestinal mixed and unclassified type GC, respectively.

MUC5AC-positive tumor cells did not necessarily express olfactomedin 4. In GI type GC, in which expression of both MUC5AC and MUC2 was observed, olfactomedin 4 expression was frequently found (Figs. 4*d–4f*). However, olfactomedin 4 was also expressed in tumor cells that did not express MUC5AC or MUC2. In total, there was no clear relationship between expression of olfactomedin 4 and the 4 gastric/intestinal markers tested (Table II). On the basis of the expression of these 4 markers, we classified the 167 GC cases phenotypically as 49 (29%) G type, 39 (23%) I type, 30 (18%) GI type, and 49 (29%) N type. Expression of olfactomedin 4 was observed more frequently in G type GC than in other (I, GI, and N) GC types ($p = 0.0058$, Fisher's exact test).

Serum olfactomedin 4 concentration in healthy subjects and noncancer and GC patients

We next examined whether olfactomedin 4 could be detected by ELISA in sera from patients with GC. Western blot analysis did not detect olfactomedin 4 protein in culture media of the MKN-1, MKN-28 and MKN-74 GC cell lines, whereas high levels of olfactomedin 4 protein were found in culture media of olfactomedin 4-transfected MKN-1 cells and the MKN-45 GC cell line (Figs. 1*a* and 1*c*). We used ELISA to test culture media from these cell lines. Olfactomedin 4 protein was detected in culture media from olfactomedin 4-transfected MKN-1 cells and MKN-45 cell lines by ELISA (Fig. 5*a*). Olfactomedin 4 protein was not detected in culture media of MKN-1, MKN-28 and MKN-74 cell lines by ELISA (Fig. 5*a*). Culture media of olfactomedin 4-transfected MKN-1 and MKN-45 cells were preabsorbed with recombinant olfactomedin 4 protein before being tested by ELISA. The specificity of olfactomedin 4 recognition was confirmed by the marked decrease in the ELISA signals after preabsorption (data not shown).

The distribution of serum olfactomedin 4 in healthy individuals, patients with chronic-active gastritis (*H. pylori*-positive) and

TABLE II – RELATIONSHIP BETWEEN OLFACTOMEDIN 4 EXPRESSION AND GASTRIC AND INTESTINAL MARKERS IN GASTRIC CANCER

	Olfactomedin 4 expression		p value[1]
	Positive	Negative	
MUC5AC			
Positive	45 (63%)	27	0.2075
Negative	49 (52%)	46	
MUC6			
Positive	9 (50%)	9	0.6204
Negative	85 (57%)	64	
MUC2			
Positive	27 (50%)	27	0.3173
Negative	67 (59%)	46	
CD10			
Positive	10 (53%)	9	0.8081
Negative	84 (57%)	64	

[1]Fisher's exact test.

patients with GC before surgery is shown in Figure 5*b*. The serum olfactomedin 4 concentration was similar between healthy individuals (n = 76, mean ± SE, 16.6 ± 1.6 ng/mL) and patients with chronic-active gastritis (n = 20, 11.8 ± 2.6 ng/mL). The serum olfactomedin 4 concentration in presurgical GC patients (n = 123, 36.3 ± 3.5 ng/mL) was significantly elevated (healthy individuals *vs.* all GC patients, p = 0.0018, Mann-Whitney U test), even at stage I (healthy individuals *vs.* stage I GC patients, p = 0.0401, Mann-Whitney U test) (Fig. 5*b*). Serum olfactomedin 4 concentrations were significantly elevated in presurgical GC patients regarding tumor stage: stage II (n = 8) 61.5 ± 21.1 ng/mL; stage III (n = 15) 45.3 ± 10.0 ng/mL and stage IV (n = 40) 36.0 ± 6.8 ng/mL.

Next, to investigate whether the olfactomedin 4 levels in serum samples are correlated with olfactomedin 4 expression levels in primary GC tissue samples, differences in the serum concentration of olfactomedin 4 between olfactomedin 4-positive cases and olfactomedin 4-negative cases which were evaluated by immunostaining were tested. Among the serum samples from the 123 GC patients, primary GC tissue samples were available for immunohistochemical analysis of olfactomedin 4 from 59 cases. In Case 38, which showed a high serum olfactomedin 4 concentration, strong and extensive olfactomedin 4 staining was observed in the primary GC sample (Fig. 5*c*). In contrast, in Case 103, although the serum olfactomedin 4 concentration was very low, strong and extensive olfactomedin 4 staining was observed in the primary GC sample (Fig. 5*c*). The olfactomedin 4 concentration in serum samples from patients with GC showing olfactomedin 4-positive immunostaining (n = 38, 40.1 ± 6.1 ng/mL) was not statistically different from that in serum samples with GC showing olfactomedin 4-negative immunostaining (n = 21, 46.9 ± 10.2 ng/mL) (p = 0.7638, Mann-Whitney U test). Because the mean ± 2SD of serum olfactomedin 4 concentration in healthy individuals was 44.3 ng/mL, the cutoff level for olfactomedin 4 was set at 44.3 ng/mL. The sensitivity and specificity for detection of GC were 31% (38/123) and 95% (72/76), respectively.

Reg IV, CA19-9 and CEA levels were also measured in the same serum samples. The sensitivity and specificity of Reg IV for detection of GC were 37% (46/123) and 97% (74/76), respectively. The sensitivity and specificity of CA19-9 for detection of GC were 12% (15/123) and 100% (76/76), respectively. The sensitivity and specificity of CEA for detection of GC were 14% (17/123) and 100% (76/76), respectively. Spearman's correlation test revealed only a weak correlation between serum olfactomedin 4 and Reg IV (r = −0.0802, p = 0.3778) or CA19-9 (r = −0.0102, p = 0.9107) (Figs. 5*d* and 5*e*). In contrast, Spearman's rank correlation test revealed significant correlations between serum olfactomedin 4 and CEA (r = 0.1897, p = 0.0356) (Fig. 5*f*). Of the GC patients with normal serum Reg IV values, 32% were found to express olfactomedin 4 at 95% specificity. Of the GC patients with normal serum CA19-9 values, 32% were found to express olfactomedin 4 at 95% specificity, and of the GC patients with normal serum CEA values, 30% were found to express olfactomedin 4 at 95% specificity. The specificities and sensitivities of serum olfactomedin 4, Reg IV, CA19-9 and CEA with respect to tumor stage are shown in Table III. In patients with stage I GC, the sensitivity of serum olfactomedin 4 (25%) was superior to that of CA19-9 (5%, p = 0.0102, Fisher's exact test) or CEA (3%, p = 0.0034, Fisher's exact test). The specificities and sensitivities of serum olfactomedin 4 combined with Reg IV, and serum CA19-9 combined with CEA, with respect to tumor stage, are shown in Table IV. In patients with stage I GC, the sensitivity of serum olfactomedin 4 combined with Reg IV (52%) was superior to that of CA19-9 combined with CEA (7%, $p < 0.0001$, Fisher's exact test).

Serum olfactomedin 4 concentration in CRC patients

We also examined whether olfactomedin 4 could be detected by ELISA in sera from patients with CRC. The serum olfactomedin 4 concentration in presurgical CRC patients (n = 50, 31.4 ± 6.6 ng/mL) was not significantly elevated (healthy individuals *vs.* all CRC patients, p = 0.3537, Mann-Whitney U test). When the cutoff level for olfactomedin 4 was set at 44.3 ng/mL, the sensitivity for detection of CRC was 20% (10/50). Reg IV, CA19-9 and CEA levels were also measured in the same serum samples. The sensitivities of Reg IV, CA19-9 and CEA for detection of CRC were 8% (4/50), 20% (10/50) and 42% (21/50), respectively. Spearman's correlation test revealed only a weak correlation between serum olfactomedin 4 and Reg IV (r = −0.0767, p = 0.5965), CA19-9 (r = −0.1087, p = 0.4524) or CEA (r = −0.0002, p = 1.0000). Of the CRC patients with normal serum Reg IV values, 20% were found to express olfactomedin 4 at 95% specificity. Of the CRC patients with normal serum CA19-9 values, 23% were found to express olfactomedin 4 at 95% specificity, and of the CRC patients with normal serum CEA values, 17% were found to express olfactomedin 4 at 95% specificity. The specificities and sensitivities of serum olfactomedin 4, Reg IV, CA19-9 and CEA with respect to tumor stage are shown in Table V. In patients with stage I, II and III CRC, the sensitivities of serum olfactomedin 4 were lower than those of CEA.

Olfactomedin 4 is frequently expressed in Reg IV-negative GC cases

Because the sensitivity of serum olfactomedin 4 combined with Reg IV for GC detection was higher than that of serum olfactomedin 4 for detection of GC, olfactomedin 4 may be frequently expressed in Reg IV-negative GC cases. Therefore, we performed immunohistochemical analysis of Reg IV in 167 GC cases. In total, 45 (27%) of 167 GC cases were positive for Reg IV. Olfactomedin 4 staining was observed more frequently in Reg IV-negative GC cases (62%) than in Reg IV-positive cases (40%, p = 0.0136, Fisher's exact test) (Table I).

Discussion

Previously, we performed SAGE on 4 primary GCs[4] and identified several GC-specific genes.[5] Of these genes, olfactomedin 4 is a candidate gene for cancer-specific expression. Although it has been reported that enhanced olfactomedin 4 expression is more frequently seen in intestinal type GC than in diffuse type GC by immunostaining,[10] the relationship of olfactomedin 4 expression to clinicopathologic characteristics or patients' survival was not investigated in GC. In this study, we generated 2 mouse monoclonal antibodies against olfactomedin 4, and performed immunohistochemical analysis. In non-neoplastic gastric mucosa, foveolar epithelium was not stained by anti-olfactomedin 4 antibody, whereas olfactomedin 4 was expressed in the basal crypt epithelium in the intestinal metaplasia. In GC, olfactomedin 4 expression was observed frequently in intestinal type GC. This is consistent with results reported previously.[10] In this study, olfactomedin

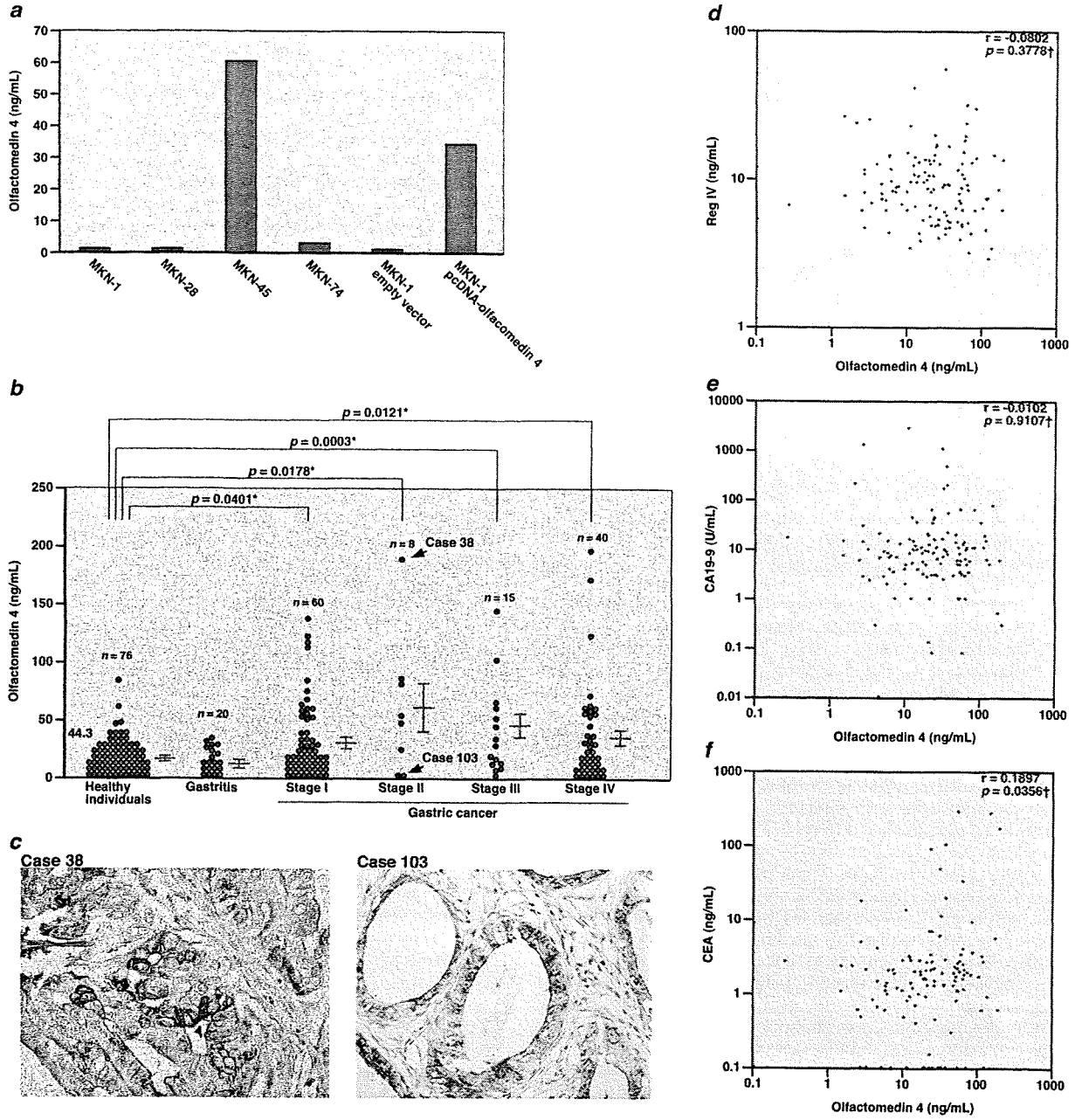

FIGURE 5 – ELISA of serum samples from patients with GC. (*a*) Detection of olfactomedin 4 in culture media by ELISA. Olfactomedin 4 was detected in culture media of MKN-45 and olfactomedin 4-transfected MKN-1 cells but not MKN-1, MKN-28, and MKN-74 cells. (*b*) Detection of olfactomedin 4 protein in serum samples by ELISA. A high concentration (44.3 ng/mL) of olfactomedin 4 was detected in 38 serum samples from patients with GC. Yellow bars indicate the cutoff levels defined in this study. Red bars indicate the means ± SE. *Mann-Whitney *U* test. (*c*) Immunostaining of olfactomedin 4 in primary GC samples. Strong and extensive olfactomedin 4 staining was observed in Case 38, which also showed high concentrations of olfactomedin 4 in serum samples. In Case 103, extensive olfactomedin 4 staining was observed in Cases 103; however, the serum concentration of olfactomedin 4 was low. (*d*) Relationship between serum concentrations of olfactomedin 4 and Reg IV. Correlation was examined using Spearman's rank correlation. (*e*) Relationship between serum concentrations of olfactomedin 4 and CA19-9. Correlation was examined using Spearman's rank correlation. (*f*) Relationship between serum concentrations of olfactomedin 4 and CEA. †Spearman's rank correlation.

TABLE III – DIAGNOSTIC SPECIFICITIES AND SENSITIVITIES OF SERUM OLFACTOMEDIN 4, REG IV, CA19-9 AND CEA WITH RESPECT TO TUMOR STAGE IN GASTRIC CANCER

	Olfactomedin 4 (%)	Reg IV (%)	*p* value[1]	CA19-9 (%)	*p* value[2]	CEA (%)	*p* value[3]
Stage I (n = 60)	25	35	0.4485	5	0.0102	3	0.0034
Stage II (n = 8)	63	50	1.0000	13	0.3330	13	0.3330
Stage III (n = 15)	40	53	0.7520	33	1.0000	40	1.0000
Stage IV (n = 40)	30	33	1.0000	15	0.2961	20	0.4628
Specificity	95	97	–	100	–	100	–

[1]Fisher's exact test. Olfactomedin 4 *vs.* Reg IV.–[2]Fisher's exact test. Olfactomedin 4 *vs.* CA19-9.–[3]Fisher's exact test. Olfactomedin 4 *vs.* CEA.

4 expression was frequently found in early stage GC. It is generally accepted that apoptosis suppresses oncogenic transformation. The ability of tumor cell populations to expand in number is determined not only by the rate of cell proliferation but also by the rate of cell attrition. Apoptosis represents a major source of this attrition.[22] Thus, resistance to apoptosis is a hallmark of most and perhaps all types of cancer. It has been reported that olfactomedin 4 interacts with GRIM-19 to attenuate retinoic acid and interferon beta-mediated cellular apoptosis and transient expression of olfactomedin 4 promotes tumor growth in C57/BL/6 mice.[9] Therefore, overexpression of olfactomedin 4 may contribute to carcinogenesis by resistance to apoptosis at least in early stage GC.

In contrast, in CRC, it has been reported that olfactomedin 4 down-regulation is found in late stage cases, and in patients with shorter survival.[12] In this study, expression of olfactomedin 4 was less frequent in late stage GC than in early stage GC. In addition, in intestinal type GC, patients with olfactomedin 4-positive GC had a better survival rate than patients with olfactomedin 4-negative GC. It has been reported that olfactomedin 4 binds to cadherin and lectins and affects cell adhesion in HEK293 cell lines. Because expression of E-cadherin was preserved in intestinal type GC, interaction between olfactomedin 4 and E-cadherin may inhibit cancer cell invasion and metastasis. In fact, forced expression of olfactomedin 4 in an HT-29 colon cancer cell line decreases cell adhesion and migration.[12] In diffuse type GC, the survival rate was not statistically different between patients with olfactomedin 4-positive GC and those with olfactomedin 4-negative GC. In diffuse type GC, E-cadherin is frequently inactivated by DNA hypermethylation,[23] suggesting that olfactomedin 4 expression may not affect the ability of cancer cell invasion and metastasis in diffuse type GC. The detailed mechanisms of inhibition of cell migration should be investigated.

Olfactomedin 4 was expressed in the intestinal metaplasia. It is known that Cdx2, a mammalian caudal-related intestinal transcription factor, is important for the maintenance of intestinal epithelial cells. Several lines of evidence have suggested that intestinal metaplasia of the stomach and I type GC are associated with ectopic Cdx2 expression.[24–27] However, olfactomedin 4 expression was frequently observed in G type GC in this study. Therefore, regulation of olfactomedin 4 expression is not simply involved in intestinal differentiation, and so the significance of olfactomedin 4 expression in intestinal metaplasia, small intestine and colon remains unclear.

Serum olfactomedin 4 is a novel biomarker for GC. Despite the reliability of CA19-9 and CEA as markers for detection of GC, CA19-9 and CEA are unsuitable for detection of early GC. In fact, in this study, CA19-9 and CEA were found in serum in 5% and 3% of patients, respectively, with stage I GC. Of 60 serum samples from patients with stage I GC, 25% showed high levels of olfactomedin 4, indicating that olfactomedin 4 is a good serum marker for early detection of GC. In our previous study, the diagnostic sensitivity and specificity of serum Reg IV for detection of GC were 36 and 99%, respectively.[7] In this study, in stage I GC cases, the sensitivity and specificity of serum olfactomedin 4 combined with Reg IV for GC detection were 52 and 95%, respectively. Therefore, serum olfactomedin 4 combined with Reg IV are likely to be suitable for early screening for GC detection. Expression of olfactomedin 4 was observed more frequently in G type GC than in other types of GC. Because expression of Reg IV is found in I type GC,[6] there is a tendency that olfactomedin 4-positive GC cases will not coincide with Reg IV-positive GC cases. In fact, olfactomedin 4-positive GC cases were found more frequently in Reg IV-negative GC cases than in Reg IV-positive cases.

In this study, we also measured olfactomedin 4 levels in the sera from patients with CRC by ELISA. Although *OLFM4* mRNA overexpression has been reported in CRC,[11] preoperative levels of olfactomedin 4 were increased in a small number of serum samples from CRC patients at stage I-III, indicating that serum olfactomedin 4 is unsuitable for early detection of CRC. The sensitivities of serum olfactomedin 4 at stage I-III was lower than those of CEA.

In this study, the olfactomedin 4 concentration in serum samples from patients with GC showing olfactomedin 4-positive immunostaining was not statistically different from that in serum samples with GC showing olfactomedin 4-negative immunostaining. This discrepancy between immunostaining and ELISA results may be due to methodologic differences. Olfactomedin 4 immunohistochemistry results were evaluated as the percentage of stained tumor cells; the intensity of immunostaining was not evaluated because we had no suitable internal control for the immunohistochemistry. More detailed quantitative methods for the measurement of olfactomedin 4 protein, such as intratumor olfactomedin 4 concentration, are needed to clarify the relationship between levels of olfactomedin 4 protein in sera and levels in primary GC samples. Furthermore, the serum concentration of olfactomedin 4 before and after surgical resection of GC should be examined.

In conclusion, our present data show that serum olfactomedin 4 in combination with Reg IV is a highly sensitive biomarker for GC patients. Although extensive staining of olfactomedin 4 was observed in GC but not in non-neoplastic gastric mucosa, in intestinal type GC, patients with olfactomedin 4-positive GC had a better survival rate than patients with olfactomedin 4-negative GC. Whether olfactomedin 4 has oncogenic or tumor suppressive properties remains unclear. Functional analysis is required to determine whether olfactomedin 4 is a therapeutic target for GC.

TABLE IV – DIAGNOSTIC SPECIFICITIES AND SENSITIVITIES OF SERUM OLFACTOMEDIN 4 COMBINED WITH REG IV AND SERUM CA19-9 COMBINED WITH CEA WITH RESPECT TO TUMOR STAGE IN GASTRIC CANCER

	Olfactomedin 4 combined with Reg IV (%)	CA19-9 combined with CEA (%)	*p* value[1]
Stage I (n = 60)	52	7	<0.0001
Stage II (n = 8)	88	13	0.1763
Stage III (n = 15)	73	47	0.5550
Stage IV (n = 40)	53	28	0.1476
Specificity	95	100	–

[1]Fisher's exact test. Olfactomedin 4 combined with Reg IV *vs.* CA19-9 combined with CEA.

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TABLE V – DIAGNOSTIC SPECIFICITIES AND SENSITIVITIES OF SERUM OLFACTOMEDIN 4, REG IV, CA19-9 AND CEA WITH RESPECT TO TUMOR STAGE IN COLORECTAL CANCER

	Olfactomedin 4 (%)	Reg IV (%)	*p* value[1]	CA19-9 (%)	*p* value[2]	CEA (%)	*p* value[3]
Stage I (n = 12)	8	8	1.0000	25	0.6000	42	0.1961
Stage II (n = 11)	36	27	1.0000	36	1.0000	45	1.0000
Stage III (n = 27)	19	0	0.0563	11	0.7085	41	0.2559

[1]Fisher's exact test. Olfactomedin 4 *vs.* Reg IV.–[2]Fisher's exact test. Olfactomedin 4 *vs.* CA19-9.–[3]Fisher's exact test. Olfactomedin 4 *vs.* CEA.

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RESEARCH COMMUNICATION

Incidence and Survival of Childhood Cancer Cases Diagnosed between 1998 and 2000 in Hiroshima City, Japan

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Abstract

There have been few studies on cancer incidence and survival among children in Japan. Childhood cancer cases in Hiroshima City can be ascertained almost perfectly in terms of completeness and validity as both a population-based cancer registry and a tissue registry cover the whole area. We report here recent incidence and survival of childhood cancer in Hiroshima City. Subjects were cancer patients less than 15 years of age in Hiroshima City registered in the Hiroshima City Cancer Registry and/or the Hiroshima Prefecture Tumor Registry (tissue registry) between 1998 and 2000. Cancer incidence in Hiroshima City was calculated for 12 diagnostic groups according to the International Classification of Childhood Cancer, and compared with general incidence in Japan. Five-year survival was calculated by the Kaplan-Meier method. There were 63 children who had a cancer newly diagnosed during 1998-2000, with only one death-certificate-only case (1.6%). Age-standardized incidence rates (per million) was 144.3 for boys and 93.9 for girls. Leukemia was the most frequent (29%) among the 12 diagnostic groups. There were 13 cancer deaths during this period and five-year survival was 79% (95% Confidence Interval: 67%-87%). Childhood cancer incidence was slightly higher than that for all of Japan, but the relative distribution of patients by diagnostic group was compatible with the general pattern. Both of these observations might be due to the high quality of the tumor and tissue registries.

Key Words: Childhood cancer - cancer registry - incidence - mortality - survival

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Introduction

The cancer registries in Hiroshima, which are the Hiroshima City Cancer Registry (HCPR) and the Hiroshima Prefecture Tumor Registry (HPTR), have been carried out by local governments and local medical associations and supported by the Radiation Effects Research Foundation (RERF). Epidemiological studies have been conducted using the cancer registry data to estimate cancer incidence and to examine several risk factors (Preston et al., 2007; 2008). Thus, it is important to know the background information of the cancer incidence and to continue to evaluate the quality of the cancer registry.

Cancer incidence in Hiroshima City is reported annually as part of the routine work of a cancer registry and has been published (Tumor Statistics Committee of Hiroshima City Medical Association, Curado et al., 2007). Cancer incidence rates (1996-2000) were relatively higher than that of other cancer registries in Japan, probably because the proportion of DCO (death-certificate-only) cases is low (3.1%) and the proportion of MV (microscopically verified) is high (84.5%) (Curado et al., 2007). Such good quality of data resulted from having both a population-based cancer registry and tissue registry which cover the whole area.

In spite of the recent high incidence rate of all ages, the childhood cancer incidence rate in Hiroshima City (1980-1989) was relatively at the same level as those among Japan (incidence rates; Hiroshima 117.1, vs. Osaka 133.4, Kanagawa 94.8, Miyagi 121.8, and Nagasaki 130.0) (Parkin et al., 1998). However, it has not been reported based on more recent data.

In this study, we describe the incidence, mortality, patterns of initial treatment, and survival among childhood cancer patients in Hiroshima City who were diagnosed in more recent years (during 1998-2000), compare the results with those in Japan and other countries, and examine various circumstances related to the childhood cancer patients.

Materials and Methods

Study area

Hiroshima City, the capital of Hiroshima Prefecture, is located in the western part of Japan and covers an area

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of 740 km^2. As is widely known, an atomic bomb was dropped on Hiroshima City in 1945. After the war, Hiroshima City has been developed as an administrative and industrial center in the Chugoku and Shikoku areas. The population in 2000 was 1.12 million, with an age distribution of 15.4% 0-14 years old, 70.3% 15-64 years old, and 14.2% 65 or more years old (Statistics Bureau et al., 2001).

Case ascertainment

The Hiroshima City Cancer Registry (HCCR), established in 1957, has adopted an active method to obtain cancer data: namely, the registry staff goes out to 16 major general hospitals around Hiroshima City to abstract cancer information from the medical charts (Nishi et al. 2008). The staff check all of the medical charts in the hospitals and, for subjects found to be diagnosed with cancer, abstract information on the details and history of the patient's cancer, motivation for the first physician visit, and initial treatment.

The Hiroshima Prefecture Tumor Registry (HPTR), initiated in 1973, collects tumor tissues on prepared slides, including both benign and malignant tissue, as well as pathology reports. The pathologists of the registry working committee summarize and code those data, based on the pathology report and the tissue slides, using the International Classification of Disease for Oncology 3rd revision (ICD-O-3) (WHO 2000). Diagnoses based on both the tissue slide and the pathology report enhances the quantitative and qualitative caliber of the cancer registry. Moreover, the HPTR has had legal authority to obtain death certificates since 1998. We used HCCR, HPTR, and death certificate data, so that we could ascertain the entire course of cancer from the first physician visit to diagnosis, treatment, and ultimate prognosis in terms of survival.

The number of study subjects was 63 cases who were living in Hiroshima City and who were diagnosed as having a first primary cancer between 1998 and 2000 at ages of 0-14 years. Only one case (1.6% of diagnoses) was identified through death-certificate-only (DCO).

Classification of Childhood Cancer

Childhood cancer needs a special classification system other than ICD-O (Ajiki et al., 2004). We adopted the International Classification of Childhood Cancer 3rd revision (ICCC-3) (Steliarova-Foucher et al. 2005) to assign incident childhood cancer cases to 12 diagnostic groups, based on the Main Classification table of SEER (Surveillance Epidemiology and End Results) Data Reporting tools (Surveillance Epidemiology and End Results).

Analyses

We calculated the age-standardized cancer incidence rate per million (standard population: the Japanese standard population in 1985) (ASR) among children aged 0-14 in Hiroshima City and examined the distribution of patients according to 12 diagnostic groups. The proportions of patients who underwent surgery, radiotherapy, and/or chemotherapy were also calculated according to the same diagnostic groups.

The HPTR has obtained the underlying cause of death among people who died and whose address was in Hiroshima Prefecture since 1998. The number of childhood cancer deaths was calculated according to underlying cause of death (ICD-10) (WHO 1992) for each year from 1998 to 2000.

Five-year cumulative survival was calculated using the Kaplan-Meier method. Five-year relative survival was also calculated by the Ederer II method using the Japanese cohort survival table downloaded from the National Cancer Center in Tokyo, Japan (Center for Cancer Control and Information Services). The single DCO case was excluded. Patients were followed for vital status until the end of July 2006 using death certificates, so all patients were followed for at least five years.

Ethical Consideration

Approval to use the data of HCCR and HPTR was obtained from each review committee. The data do not include identifying information such as the patient name and address.

Results

There were 63 children (0-14 years of age) with malignancies diagnosed between 1998 and 2000 in Hiroshima City. The cases comprised 24 in 1998, 21 in 1999, and 18 in 2000. Twenty-three cases (36%) had cancer information from both HCCR and HPTR, 33 (52%) had information from only HCCR, 6 (10%) had information from only HPTR, and one case (1.6%) was DCO.

The ASR of all types of childhood cancer was 144.3 per million for boys and 93.9 per million for girls (Table 1). The distribution of patients by diagnostic group is shown in the same table. Among childhood cancers for boys, leukemia was the most prevalent (30.8%), followed

Table 1. Number of Incident Cases and Age Standardized Incidence Rate among Childhood Cancer Patients in Hiroshima City, 1998-2000

Cancer site/type	Boys		Girls	
	N (%)	Rate/10^5	N (%)	Rate/10^5
I. Leukemia	12 (31)	44.5	6 (25)	23.5
II. Lymphoma and reticuloendothelial system	5 (13)	18.6	2 (8)	7.8
III. Central nervous system and intracranial and intraspinal	4 (10)	14.8	5 (21)	19.7
IV. Sympathetic nervous system (neuroblastoma)	4 (10)	14.9	4 (17)	15.6
V. Retinoblastoma	1 (3)	3.6	2 (8)	7.8
VI. Kidney	2 (5)	7.4	1 (4)	3.8
VII. Liver	3 (8)	11.1	1 (4)	4.0
VIII. Bone	0 (0)	0.0	1 (4)	3.8
IX. Soft tissue sarcoma	3 (8)	11.1	1 (4)	3.9
X. Embryonal/gonadal	4 (10)	14.7	1 (4)	3.8
XI. Carcinoma and other malignant epithelial tumor	1 (3)	3.7	0 (0)	0.0
XII. Others/unclassified	0 (0)	0.0	0 (0)	0.0
Total	39 (100)	144.3	24 (100)	93.9

Table 2. Numbers of Patients (%) Treated with Surgery, Radiotherapy, or Chemotherapy

Cancer site/type	Surgery			Radiotherapy			Chemotherapy			Total
	Yes	No	Unknown	Yes	No	Unknown	Yes	No	Unknown	
I. Leukemia	0 (0)	17 (94)	1 (6)	0 (0)	17 (94)	1 (6)	17 (94)	0 (0)	1 (6)	18 (100)
II. Lymphoma and reticuloendothelial system	5 (71)	1 (14)	1(14)	0 (0)	6 (86)	1 (14)	4 (57)	2 (29)	1 (14)	7 (100)
III. Central nervous system tumor and intracranial and intraspinal	9(100)	0 (0)	0 (0)	4 (44)	4 (44)	1 (11)	3 (33)	5 (56)	1 (11)	9 (100)
IV. Sympathetic nervous system tumor (neuroblastoma)	8(100)	0 (0)	0 (0)	0 (0)	8(100)	0 (0)	2 (25)	6 (75)	0 (0)	8 (100)
V. Retinoblastoma	2 (67)	1 (33)	0 (0)	0 (0)	3(100)	0 (0)	2 (67)	1 (33)	0 (0)	3 (100)
VI. Kidney	3(100)	0 (0)	0 (0)	1 (33)	2 (67)	0 (0)	2 (67)	1 (33)	0 (0)	3 (100)
VII. Liver	1 (25)	2 (50)	1(25)	0 (0)	3 (75)	1 (25)	3 (75)	0 (0)	1 (25)	4 (100)
VIII. Malignant bone	1(100)	0 (0)	0 (0)	0 (0)	1(100)	0 (0)	1(100)	0 (0)	0 (0)	1 (100)
IX. Soft tissue sarcoma	4(100)	0 (0)	0 (0)	0 (0)	3 (75)	1 (25)	2 (50)	1 (25)	1 (25)	4 (100)
X. Embryonal and gonadal	3 (60)	2(40)	0 (0)	3 (60)	1 (20)	1 (20)	3 (60)	1 (20)	1 (20)	5 (100)
XI. Carcinoma/other epithelial	1(100)	0 (0)	0 (0)	0 (0)	0 (0)	1(100)	0 (0)	0 (0)	1(100)	1 (100)
Total	37 (59)	23 (37)	3 (5)	8 (13)	48 (76)	7 (11)	39 (62)	17 (27)	7 (11)	63 (100)

Table 3. Number of Childhood Cancer Deaths in Hiroshima City (1998-2000)

ICD-10	1998			1999			2000			Total		
	B	G	T	B	G	T	B	G	T	B	G	T
C22 Malignant neoplasms of liver and intrahepatic bile ducts	0	0	0	1	0	1	1	0	1	2	0	2
C56 Malignant neoplasms of ovary	0	0	0	0	0	0	0	1	1	0	1	1
C74 Malignant neoplasms of adrenal gland	0	0	0	1	0	1	1	0	1	2	0	2
C91 Lymphoid leukemia	2	1	3	0	1	1	1	0	1	3	2	5
C92 Myeloid leukemia	0	0	0	1	1	2	0	0	0	1	1	2
C95 Leukemia of unspecified cell type	0	0	0	1	0	1	0	0	0	1	0	1
C00-C97	2	1	3	4	2	6	3	1	4	9	4	13

by lymphoma and reticuloendothelial system neoplasm (12.8%). Central nervous system and intracranial and intraspinal neoplasm, sympathetic nervous system tumor (neuroblastoma), and embryonal and gonadal tumor were the third most common cancers (10.3%). As for girls, leukemia was the most common (28.6%), followed by central nervous system and intracranial and intraspinal neoplasm (14.3%), and lymphoma and reticuloendothelial system neoplasm (11.1%).

The age distribution of the patients at the time of diagnosis is shown Figure1. Out of 12 patients, less than one year old (infants), seven were diagnosed as having neuroblastoma. Among patients aged 1-4 years, the most frequent cancers were lymphomas (five cases).

Among those aged 5-9 years, there were six leukemia cases and four soft tissue sarcoma cases.

Among those aged 10-14, there were six cases of leukemia and three cases each of central nervous system and intracranial and intraspinal neoplass, and embryonal and gonadal tumors.

Treatments received by the patients are shown in Table 2. Thirty-seven children (58.7%) underwent surgery, eight children (12.7%) received radiotherapy, and thirty-nine children (61.9%) received chemotherapy. In particular, 94% of leukemia patients received chemotherapy. All eight patients (100%) with neuloblastoma underwent surgery.

Table 3 shows the number of childhood cancer deaths from 1998 to 2000: three in 1998, six in 1999, and four in 2000 for both sexes combined. During this period, lymphoid leukemia deaths were most frequent (38%).

Figure 2 shows the five-year cumulative survival among the childhood cancer patients diagnosed during 1998-2000. The five-year survival was 79% (95% confidence interval, CI: 67% - 87%) and the relative five-year survival rate was also 79%.

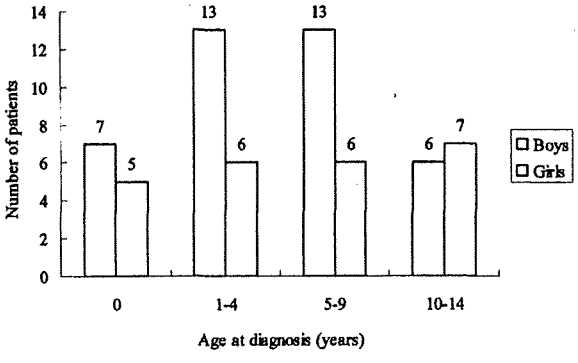

Figure 1. Distribution of Patients by Age at Diagnosis

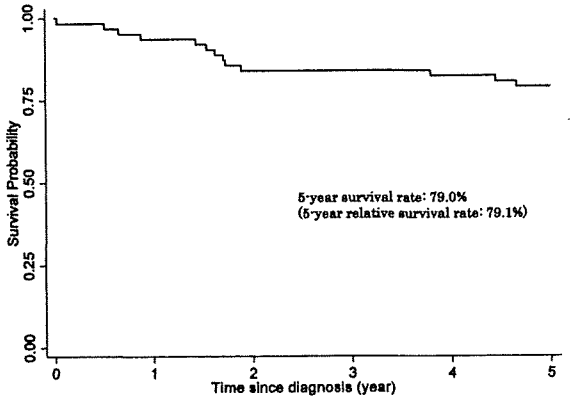

Figure 2. Kaplan-Meier Survival Probability

Discussion

This is the first report on incidence and survival among childhood cancer patients in Hiroshima City. Proportions of type-specific cancers in Hiroshima City were similar to those in Japan overall, which were (in order, beginning with the most frequent): leukemia (boys, 34%; girls, 34%), central nervous system (CNS) and miscellaneous intracranial and intraspinal neoplasma (brain and CNS) (boys, 15%; girls, 11%), and neuroblastoma and other peripheral nervous cell tumors (neuroblastoma) (boys, 13%; girls, 11%). However, the total childhood cancer ASR was higher than those which were 103.7 per million for boys and 80.1 per million for girls in 2000 estimated overall in Japan (Marugame et al., 2007). The percentage of DCO cases in Hiroshima City was much lower (1.6%) than that in Japan overall (7.1%). Moreover, comparing with the childhood cancer incidence rate (1980 - 1989) among other cancer registries in Japan, those of Hiroshima City (117.1 per million), Osaka (133.4), Miyagi (121.8), and Nagasaki (130.0) were almost the same level, while that of Kanagawa Prefecture was only low (94.8). It is considered that the percentage of DCO cases in Kanagawa Prefecture was 30 %, in spite of other cancer registries the percentage of DCO cases were less than 1% (Parkin et al., 1998). Thus, the apparently higher incidence in Hiroshima (1998-2000) may be due to the quality of its cancer registry resulting in a greater number of acceptable diagnoses.

All patients who were suspected of having neuroblastoma by mass screening at ages less than one year survived at least five years after diagnosis. Five-year survival for all neuroblastoma patients was 88%, which is high compared with that of the ACCIS study in the EU (59%, 1988-1997) (Spix et al., 2006) and US SEER (66.0%, 1985-1999) (Desandes et al., 2008; Surveillance Epidemiology and End Results, 2008). A higher rate of diagnosis of neuroblastoma due to mass screening has been thought to greatly affect an apparent increase in its incidence and survival (Ajiki et al., 1998; Honjo et al. 2003). Mass screening for neuroblastoma was performed for infants through six months of age until 2003 in Japan, and the seven infants who were diagnosed as having neuroblastoma were all initially identified through mass screening. In this study, among eight patients with neuroblastoma, one had a localized tumor, one had a tumor that had advanced into the regional lymph node, one had a tumor that had advanced into the regional organs, three had metastasis, and two had unknown stage; all underwent surgery. Because it is well known that a localized neuroblastoma diagnosed before the age of one has a good prognosis (Hiyama et al., 2008), surgery may be unnecessary for patients with localized tumors. Due to the fact that mass screening for neuroblastoma in Japan ceased in 2003, effects on the subsequent epidemiology of childhood neuroblastoma, such as incidence, mortality, survival, and method of treatment (surgery, chemotherapy, and radiotherapy), clearly need to be evaluated periodically.

There were about five childhood cancer deaths in Hiroshima City each year. Although it is a fact that some cancer deaths are unavoidable, from the standpoint of public health, it is necessary to continue evaluating and improving the medical management of childhood cancer patients. Issues to be evaluated include patterns of diagnosis, treatment, and reference to larger hospitals that, unlike local clinics, have the resources to provide and combine many specialties in the treatment of childhood cancer. We will also continue to gather information on children who died from cancer causes to facilitate ongoing evaluation of childhood cancer mortality (Tsutsui et al., 2009).

Five-year relative survival among Hiroshima City children was 79%, which is slightly high compared with that in Osaka (71.7%, 1990-94) (Ajiki et al., 2004), France (75.2%, 1990-1999) (Goubin et al., 2006), the ACCIS study in the EU (72%, 1988-1997), and SEER (74.0%, 1990-1999), but may not be statistically significant because the confidence interval overlapped the five-year survival of other countries. After excluding patients diagnosed with neuroblastoma, the five-year relative survival was 78%. Survival from childhood cancer has improved recently, particularly due to improvements in chemotherapy, which is used in 61% of childhood cancer cases. Therefore, because the period of diagnoses reported for Hiroshima City is later than those referenced above for other registries, increased survival due to improvements in treatment could explain some of the difference. In Hiroshima City, the reference system—whereby children suspected of having cancer are referred to a hospital specializing in cancer care or to a large general hospital to be diagnosed and undergo treatment—works quite well: 81% of the patients were treated in designated cancer-care hospitals (Sugiyama et al., 2008). It is plausible that the better survival may be due to this concentration of childhood cancer treatment in cancer-care hospitals.

Incidence of childhood cancer is substantially lower than that of adult cancer. In this study, based on a relatively small population and short follow-up period, it was difficult to evaluate the childhood cancer statistics from many traditional epidemiologic viewpoints (e.g., trends in age-specific incidence, relative proportions according to stage, and survival by diagnostic category). Furthermore, there are many important issues to be evaluated using cancer registry data, such as neuroblastoma mass screening (Ajiki et al., 1998; Hiyama et al., 2008) and childhood cancer survival as an indicator of improvements in treatment (Honjo et al., 2003; Pession et al. 2008; Swaminathan et al., 2008). Thus, it is important to report descriptive statistics on childhood cancer, and we hope to update such reports periodically.

In conclusion, the ASR (per million) was 144.3 for boys and 93.9 for girls and five year survival rate was 79% in Hiroshima City during 1998-2000. Based on childhood cancer statistics in Hiroshima City in 1998-2000 that suggest a higher incidence than in Japan overall, we infer that the high quality of the tumor and tissue registries leads to better ascertainment - thus a lower frequency of DCO diagnoses - and therefore only the appearance of higher incidence. We will continue the follow-up and

report updated childhood cancer statistics in the near future.

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Review Article

Transcriptome dissection of gastric cancer: Identification of novel diagnostic and therapeutic targets from pathology specimens

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Gastric cancer is the fourth most common malignancy in the world, and mortality due to gastric cancer is second only to that from lung cancer. 'Transcriptome dissection' is a detailed analysis of the entire expressed transcripts from a cancer, for the purpose of understanding the precise molecular mechanism of pathogenesis. Serial analysis of gene expression (SAGE) is a suitable technique for performing transcriptome dissection. Gastric cancers of different stages and histology were analyzed on SAGE, and one of the largest gastric cancer SAGE libraries in the world was created (GEO accession number GSE 545). Through SAGE, many candidate genes have been identified as potential diagnostic and therapeutic targets for the treatment of gastric cancer. Regenerating islet-derived family, member 4 (*Reg IV*) participated in 5-fluorouracil (5-FU) resistance and peritoneal metastasis, and its expression was associated with an intestinal phenotype of gastric cancer and with endocrine differentiation. *GW112* expression correlated with advanced tumor stage. Measurement of Reg IV and GW112 levels in sera indicated a sensitivity of 57% for detection of cancer. SPC18 participated in tumor growth and invasion through transforming tumor growth factor-α upregulation. Palate, lung, and nasal epithelium carcinoma-associated protein (PLUNC) was a useful marker for gastric hepatoid adenocarcinoma. Expression of *SOX9*, *HOXA10*, *CDH17*, and loss of claudin-18 expression were associated with an intestinal phenotype of gastric cancer. Information obtained from transcriptome dissection greatly contributes to diagnosis and treatment of gastric cancer.

Key words: claudin-18, gastric cancer, GW112, PLUNC, Reg IV, serial analysis of gene expression, SOX9, SPC18, transcriptome dissection

'Molecular pathology' encompasses morphopathological genomics to comprehensively understand abnormalities in morphology and function of genes and molecules. We have been studying novel diagnostic and therapeutic targets through global gene expression, epigenetics, genetic polymorphisms and their predispositional effect towards gastric cancer.[1,2] It is important to understand the pathology of cancer in order to facilitate proper medical care, with knowledge of both morphological and molecular abnormalities, and to show concretely what can be done using this knowledge of pathology, to make potential clinical applications a reality.

Although gastric cancer incidence decreases through westernization of eating habits and a decrease in *Helicobacter pylori* infection, it is the fourth most common malignancy in the world and approximately 900 000 people suffer from gastric cancer every year.[3] Mortality due to gastric cancer is second only to that from lung cancer. Although the prognosis for patients with early gastric cancer has been prolonged drastically by current methods of diagnosis and treatment, that for advanced cancer remains poor. In gastric cancer patients of all stages, the 5 year survival rate after diagnosis is around 50% in Japan, and is ≤30% in other countries.[3] Therefore, areas that need attention for better treatment of gastric cancer are: detection at an early stage; and effective medical treatments for advanced cancers. For these purposes, novel diagnostic and therapeutic targets are required to be found.

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In the history of the Japanese Society of Pathology, the report of the Japan Pathology Award lectures (formerly the homework reports) on gastric cancer was initially presented by Kunio Ota (Tokyo Medical and Dental University), 'Pathogenesis of gastric cancer' in 1964; and was followed by Tateo Nagayo (Aichi Cancer Center), Setsuya Fujita (Kyoto Prefectural University of Medicine), Eiichi Tahara (Hiroshima University), and Masae Tatematsu (Aichi Cancer Center). At the 81st Annual Meeting of the Japanese Society of Pathology held in Sendai in 1992, the Japan Pathology Award lecture was presented by Tahara, who reported that through molecular pathology, different genetic pathways of stomach carcinogenesis might be discerned for poorly differentiated and well-differentiated gastric cancers.[4,5]

After this lecture the molecular basis of gastric cancer has been studied intensively and the recent understanding of the molecular events involved in stomach carcinogenesis can be summarized as follows.[1,6] A variety of genetic and epigenetic alterations occur during multistep stomach carcinogenesis. These include activation of oncogenes and growth factors/receptors, inactivation of tumor suppressor genes, DNA repair genes and cell adhesion molecules, and abnormalities in cell cycle regulators and so on. The genetic alterations that are found in gastric cancer are gene amplification, point mutation and loss of heterozygosity, while representative epigenetic changes are gene silencing by DNA methylation and histone modification. Genetic polymorphism can predispose an endogenous cause and increase susceptibility to cancer. Some of these changes occur commonly in both differentiated and undifferentiated types, and some differ depending on the histological type or mucin phenotype (gastric vs intestinal). Many of these abnormalities have been applied to molecular diagnosis[7–9] but there may be important abnormalities that have not yet been clarified, and these may become new targets for diagnosis and treatment.

In this report we describe a method for transcriptome dissection, novel cancer-specific genes identified through global analysis of gene expression, the significance of these genes in diagnosis and treatment and in stomach carcinogenesis and differentiation.

TRANSCRIPTOME DISSECTION

'Transcriptome dissection' is a term we created to describe 'a detailed analysis of entire transcripts in affected tissues, to understand the precise molecular mechanisms of pathogenesis'. Using this approach, new candidate genes for diagnosis, treatment and prevention are able to be discovered that build a bridge to clinical applications from basic research.

As a method of transcriptome dissection, serial analysis of gene expression (SAGE) was utilized as a powerful technique that allows global analysis of gene expression in a quantitative manner, without a prior knowledge of the exact sequence of the genes.[10] SAGE is based on the following principles. A short nucleotide sequence tag (approx. 10 bp) is sufficient to uniquely identify a transcript, provided it is isolated from a defined position within the transcript. Concentration of short sequence tags allows the efficient analysis of transcripts in a serial manner through the sequencing of multiple tags within a single clone. Because the SAGE tag numbers directly reflect the abundance of mRNA, SAGE data are highly accurate and quantitative. Completion of the human genome sequence has facilitated the mapping of specific genes to individual tags. Now, the SAGEmap database includes approximately 400 SAGE libraries online and is available to the public (http://www.ncbi.nlm.nih.gov/SAGE/). The advantage of SAGE is that we can study expression of genes of interest in other tissues registered in the database. Four SAGE studies of gastric cancer have been so far reported.[11–14] We examined five samples of gastric cancer of different stage and histology from four patients and uncovered a total of 137 706 tags including 38 903 unique tags.[14] This SAGE library is one of the largest gastric cancer libraries in the world and the sequence data are publicly available at SAGEmap (GEO accession number GSE 545, SAGE Hiroshima gastric cancer tissue). Many of the genes specifically upregulated or downregulated in cancer were found by comparison of SAGE libraries from gastric cancer against normal tissue. In combination with quantitative reverse transcription–polymerase chain reaction (RT-PCR), immunohistochemistry and other methods, several candidates for novel metastasis-related genes, tumor suppressor genes and serum tumor markers were identified.[14,15]

An advantage of quantitative SAGE data is that they can be compared with other samples (not just normal tissue control). In silico analysis was performed to detect any difference in molecular bases of gastric cancer from the East and the West. Our libraries were compared with the libraries from the West that were produced by El-Rifai *et al.* as part of the Cancer Genome Anatomy Project.[11,14] A clear separation was found between the East and West tumor libraries, which had 54 differentially expressed tags.[16] These may contribute to the geographical differences in incidence and possible biological behavior of gastric cancer, and differences found in outcome for patients.

Figure 1 illustrates the strategy of clinical application from transcriptome dissection.[1,6] From detailed gene expression information, specifically upregulated or downregulated genes in a cancer can be identified. The expression of these genes is confirmed in a large number of cases on quantitative RT-PCR and immunohistochemistry. If the specific gene encodes a secretory protein, this may be detected in the blood and should be a novel serum marker of gastric cancer.

For such a molecule, a simple measuring system such as ELISA can be established for blood samples, which can then be applied for early cancer detection. After functional analysis we can then know whether the genes are novel targets that could potentially be used for molecular target therapy. Polymorphisms of genes can highly alter their expression in cancer, and may be novel risk factor candidates, and this information could possibly be used for personalized cancer prevention. A custom-made complementary DNA (cDNA) microarray with the specific genes identified on SAGE, known cancer-related genes, and known genetic markers for chemosensitivity, would be a useful tool to obtain information on biological behavior from, and sensitivity to, therapy in the clinical setting.

A practical approach is introduced here to identify cancer-specific genes, and candidate diagnostic and therapeutic targets using a SAGE database search.[17] If a gene participates in tumor progression and is specifically expressed in cancerous but not in normal tissues, the gene could be not only a cancer-biomarker, but also a possible therapeutic target, treatment of which might have minimal adverse effects. By comparing SAGE libraries of gastric cancer with those of various normal tissues in the SAGEmap database, 54 genes were identified in the gastric cancer libraries that were not present in the libraries from 14 normal tissues including brain, lung, heart, liver, kidney and so on.[17] Expression of these genes was then confirmed in tissue samples from gastric cancers and normal human organs on quantitative RT-PCR. Representative results are shown in Fig. 2. Nine genes including APin protein (*APIN*), taxol resistance-associated gene 3 (*TRGA3*), cytochrome P450, family 2, subfamily W, polypeptide 1 (*CYP2W1*), melanoma inhibitory activity (*MIA*), matrix metalloproteinase-10 (*MMP-10*), dickkopf homolog 4 (*DKK4*), *GW112*, regenerating islet-derived family, member 4 (*Reg IV*), and HORMA domain-containing 1 (*HORMAD1*) were found to have gastric cancer-specific expression.

NOVEL TARGETS FOR DIAGNOSIS AND TREATMENT OF GASTRIC CANCER

New candidate genes, *Reg IV*, *GW112*, *MMP-10* and *SPC18*, identified from transcriptome dissection will be described in terms of diagnostic and therapeutic targets.

Reg IV

Reg IV belongs to the calcium-dependent lectin superfamily, and was isolated as a gene upregulated in inflammatory bowel diseases in 2001.[18] Overexpression of *Reg IV* has been detected in colorectal carcinoma and adenoma on RT-PCR and *in situ* hybridization,[19,20] and it has been reported that high *Reg IV* expression is associated with 5-fluorouracil (5-FU) resistance in a colon cancer cell line.[19] When this analysis was begun, the expression and distribution of *Reg IV* in human tumors remained unclear, and it was assumed that it possibly participates in defense against mucosal injury, and proliferation of mucosal epithelia.

Additional study of *Reg IV* expression on quantitative RT-PCR showed that *Reg IV* was also expressed in colon cancer, but not in lung cancer and breast cancer.[21] Normal gastrointestinal tract and pancreas tissue expressed *Reg IV* at low levels, indicating that *Reg IV* was not truly cancer specific. On immunohistochemistry it was found that in non-neoplastic tissues, Reg IV was expressed in beta cells of the endocrine pancreas, goblet cells in intestinal metaplasia of the stomach, and neuroendocrine cells of the small intestine (Fig. 3a,d).[21] Twenty to thirty percent of gastric, colorectal and pancreatic cancers were positive for Reg IV expression, as were most gastrointestinal carcinoids. In accordance with quantitative RT-PCR, expression in all breast and lung cancer samples was negative. In gastric cancer, two staining patterns were noted: mucin-like staining and perinuclear staining (Fig. 3c,d). The former pattern might be associated with intestinal differentiation, and the latter might be associated with neuroendocrine differentiation. These will be discussed later.

Concerning the biological role of Reg IV, forced expression of *Reg IV* in TMK-1 gastric cancer cells inhibited 5-FU-induced apoptosis through activation of epidermal growth factor receptor (EGFR), induction of Bcl-2 and cytochrome c, and inhibition of caspase-9 and -3.[22] Therefore, Reg IV may serve as an indicator of the resistance of a cancer to 5-FU-based chemotherapy. The expression of Reg IV was then examined on immunostaining in samples from recurrent gastric cancer patients who had been treated with a combination chemotherapy of low-dose 5-FU and cisplatin. As predicted, all Reg IV-positive patients had 'no change' or 'progressive disease'.[22] Furthermore, it was found that Reg IV promoted peritoneal dissemination metastasis in a mouse model.[23] *Reg IV*-transfected MKN-28 gastric cancer cells were inoculated into the peritoneal cavity of nude mice. Number and size of metastatic tumors were higher in *Reg IV* transfectants than in controls. In this system the expression of the phosphorylated form of the epidermal growth factor (EGF) receptor, Bcl-2, Bcl-XL, survivin, and the phosphorylated form of v-akt murine thymoma viral oncogene homolog (AKT) was upregulated. This peritoneal metastasis was inhibited by treatment with *Reg IV*-small interfering RNA (siRNA). In the clinical specimens it was confirmed that the *Reg IV* levels were high in gastric cancer patients with peritoneal metastasis, and *Reg IV*-positive gastric cancer patients had poorer prognosis than *Reg IV*-negative patients.[24] Overall these findings indicate that Reg IV is a novel diagnostic tool, and a potential therapeutic target for the treatment of gastric cancer.

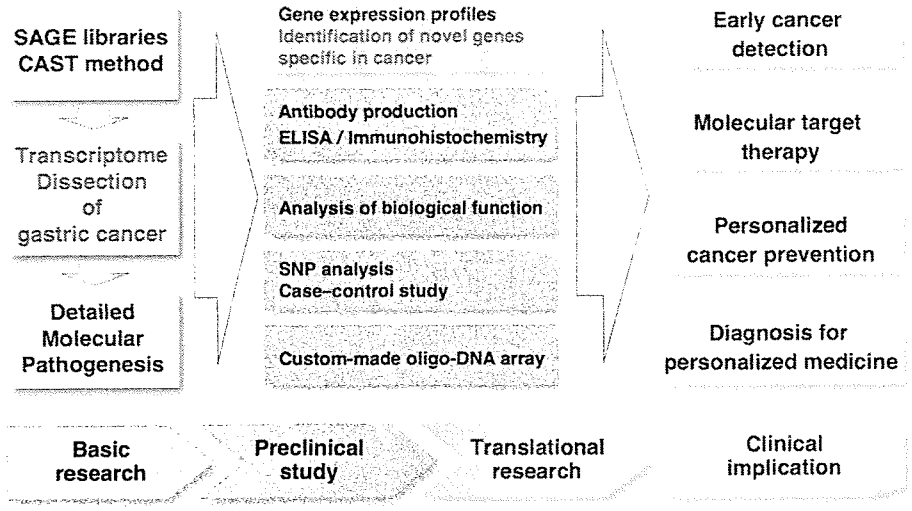

Figure 1 Strategy to search for novel genes of gastric cancer through transcriptome dissection and its clinical implication. CAST, *Escherichia coli* ampicillin trap; SAGE, serial analysis of gene expression; SNP, single nucleotide polymorphism.

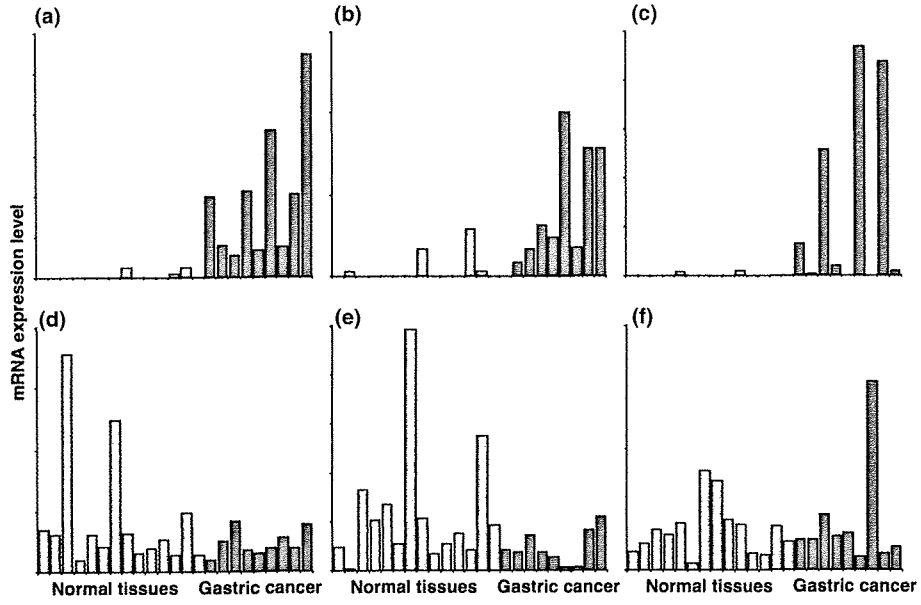

Figure 2 Representative results of quantitative RT-PCR of various normal tissues (brain, spinal cord, heart, skeletal muscle, lung, stomach, small intestine, colon, liver, pancreas, kidney, bone marrow, spleen, leukocytes) and gastric cancers. mRNA expression level of (**a**) matrix metalloproteinase-10 (*MMP-10*), (**b**) *GW112* and (**c**) taxol resistance-associated gene 3 (*TRAG3*) were much higher in gastric cancers than in normal tissues. These three are indicative of expression of genes detected in the SAGE libraries of gastric cancer-specific gene. (**d**) arginyltransferase 1 (*ATE1*); (**e**) bromodomain containing 4 (*BRD4*); (**f**) v-ets erythroblastosis virus E26 oncogene homolog 2 (avian) (*ETS2*).

Analysis of the amino acid sequence of the Reg IV protein suggested that it should be secreted.[14] It was confirmed on western blotting that native Reg IV was present in the culture media of gastric cancer cells that expressed high levels of *Reg IV* mRNA (Fig. 4) and that V5-tagged Reg IV protein was detected in not only cell extracts, but also culture media from a Reg IV-V5-expressing gastric cancer cell line, but not control cells. Although early detection is especially important for the treatment of gastric cancer, no good serological marker exists for detection of early cancer.[25] Tests for known tumor markers such as CEA and carbohydrate antigen (CA) 72-4 do not have satisfactory sensitivity for early detection, although they may have prognostic impact. Reg IV levels in the sera of gastric cancer patients and healthy controls were then measured on ELISA to determine whether Reg IV is a serum tumor marker or not. Serum levels of Reg IV protein were similar between the healthy controls and patients with chronic active gastritis, and serum Reg IV levels were significantly elevated in gastric cancer patients, even at stage I.[22] If the cut-off level was set at 2 ng/ml, specificity was 99%, and diagnostic sensitivity was 36%, which is much superior to that of serum CEA (14%) or CA19-9 (15%).

GW112

GW112, also called olfactomedin 4 (*OLFM4*) or human G-CSF clone-1 (*hGC-1*), was originally cloned from human myeloid cells and encodes a secreted glycoprotein of 510 amino acids.[26] GW112 is normally expressed in the bone marrow, intestine and prostate, and altered expression is observed in various cancers including those of the colon,

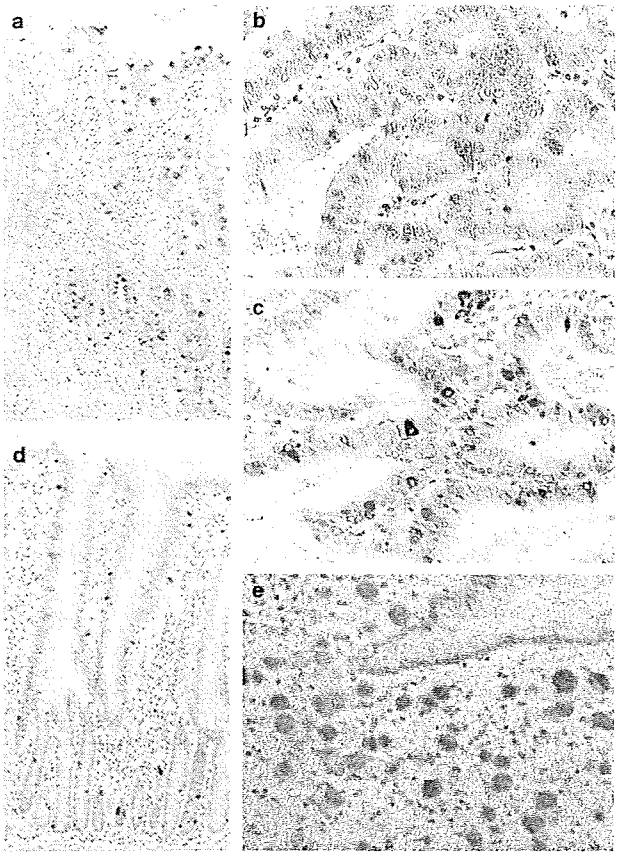

Figure 3 Immunostaining of regenerating islet-derived family, member 4 (Reg IV) in non-neoplastic tissues and gastric cancers. Reg IV is expressed (**a**) in goblet cells of intestinal metaplasia of the stomach and (**d**) neuroendocrine cells of the small intestine. (**b**,**c**,**e**) Strong expression is observed in gastric cancers. There are two staining patterns: (**b**) mucin-like staining and (**c**) perinuclear staining. (**e**) Gastric signet-ring cell carcinoma is also positive for Reg IV.

breast, and lung cancers.[27,28] Because GW112 interacts with GRIM19, cadherin and lectins, GW112 may facilitate apoptosis, tumor growth and invasion.[29,30]

On quantitative RT-PCR low levels of *GW112* expression were detected in some normal tissues, while strong expression was detected in gastric cancers.[17,31] Fifty-eight percent of gastric cancers had overexpression of *GW112* associated with stage III and IV cancers. A monoclonal antibody was then produced for GW112, and immunohistochemistry undertaken. GW112 was expressed in epithelial cells at the bottom of the intestinal crypt and intestinal metaplasia of the stomach, and strong GW112 expression was detected in 60% of gastric cancers (Fig. 5). Significant association was found between GW112 expression and intestinal histology. Furthermore, a significant inverse correlation was detected between Reg IV protein level and that of GW112 in gastric cancer.

An ELISA system was established to measure serum levels of these proteins. Approximately 30% of gastric cancer

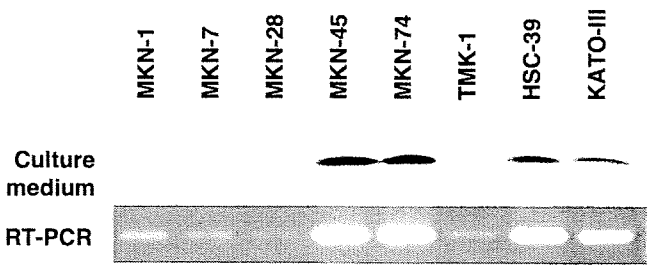

Figure 4 Detection of regenerating islet-derived family, member 4 (Reg IV) in culture media of gastric cancer cell lines. Western blot analysis of culture media of eight gastric cancer cell lines and northern blot analysis of cell extracts are shown. Reg IV protein was detected in culture media of cell lines that express *Reg IV* mRNA at high levels. RT-PCR, reverse transcription–polymerase chain reaction.

patients displayed high levels of GW112 in their sera regardless of tumor stage, even at stage I. Specificity of the test was 95%, indicating that GW112 is also a good serum tumor marker for gastric cancer. Importantly, no correlation was detected between Reg IV and GW112 levels. Measurement of Reg IV and GW112 indicated a sensitivity of 57% for detection. A combination of Reg IV and GW112 may serve as a highly sensitive biomarker for gastric cancer. Clinical application is expected.

MMP-10

MMP-10, also known as stromelysin 2, is one of the cancer-specific genes identified using the same method as that for *Reg IV* and *GW112* (Fig. 2).[17] Among the nine cancer-specific genes identified, *MMP-10* was most frequently overexpressed in gastric cancer. MMP induce extracellular matrix breakdown, associated with tissue destruction during cancer invasion and metastasis.[32] Overexpression of MMP-10 has been reported in various cancers such as cancers of the lung, esophagus and liver. Our immunohistochemical study demonstrated MMP-10 to be correlated with tumor progression and a poor prognosis for gastric cancer patients. Importantly, high levels of MMP-10 protein were detected in serum samples from >90% of gastric cancer patients, regardless of tumor stage, while diagnostic specificity was 85%.[17] In samples from patients with stage I gastric cancer, 89% showed high serum levels of MMP-10. Therefore, MMP-10 is extremely useful for screening and early detection of gastric cancer.

SPC18

Signal peptidase complex 18KDa (*SPC18*) is another new therapeutic candidate gene identified through transcriptome

dissection. As already mentioned, SAGE is a powerful technique for performing a global analysis of gene expression in a quantitative manner. It is difficult, however, to apply SAGE in the clinical setting to study large numbers of clinical samples because mass sequencing is required. In contrast, microarrays are a conventional technique for examining the expression of large numbers of genes at the same time. A custom-made microarray for the study of stomach carcinogenesis and possible future clinical application was prepared. The microarray, named Ex-STOMACHIP, consisted of 395 genes (478 cDNAs) and contained: genes selected as the 20 most upregulated and downregulated tags in SAGE libraries of gastric cancer; genes known to participate in carcinogenesis; and genes related to metastasis and chemosensitivity identified from other microarray studies.[15] Using Ex-STOMACHIP in combination with quantitative RT-PCR on 42 samples of gastric cancer, *SPC18* was identified as one of the significantly upregulated genes in stage III/IV gastric cancer compared with stage I/II.

Secretory proteins are usually synthesized as precursors with signal peptides that are cleaved by a family of signal peptidases following membrane translocation.[33] Signal peptidase complex (SPC) has five distinct subunits, and SPC18 and SPC21 are presumed to have catalytic activity.[34,35] Substrate specificity, however, remains unknown and no study has been reported on their expression and role in cancer. Quantitative RT-PCR demonstrated that *SPC18* was overexpressed in 40% of gastric cancer samples, and the overexpression was significantly associated with advanced tumor stage and presence of lymph node metastasis.

An expression vector containing *SPC18* was constructed and introduced into the MKN-1 gastric cancer cell line, which has a low level of *SPC18* expression. Under a condition of 1% serum, *SPC18* significantly stimulated cell proliferation, measured on 3-(4,5-dimethylthiaol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. *SPC18* was also found to stimulate cell migration and invasion, monitored by wound healing assay and matrigel invasion assay. The important question is what the target secretory proteins of the signal peptidase SPC18 are, in relation to tumor growth and invasion. The level of transforming growth factor (TGF)-α in the culture media was measured and found to be increased 10–20-fold in *SPC18*-transfected MKN-1 cells in comparison with control MKN-1 cells. The same pattern was observed for EGF levels in the culture media. Furthermore, *SCP18*-siRNA treatment specifically reduced the levels of TGF-α and EGF in SPC18-producing KATO-III gastric cancer cells. Therefore, TGF-α and EGF might be important targets for SPC18. It was also confirmed that *SPC18* stimulated tumor growth in SCID mice. EGFR was activated in the formed tumor with forced expression of *SPC18*. Thus, SPC18 promoted TGF-α/EGF secretion possibly by processing these precursors, resulting in EGFR activation and stimulation of tumor growth and invasion. Therefore, SPC18 participates in the growth and invasion of tumors, partly through TGF-α or EGF upregulation, and may serve as a marker of high-grade malignancy, as well as a novel therapeutic target.

APPLICATION TO HISTOPATHOLOGICAL DIAGNOSIS

Here Reg IV and palate, lung, and nasal epithelium carcinoma-associated protein (PLUNC) are provided as an example of candidates identified from transcriptome dissection used for histopathological diagnosis.

Reg IV

Signet-ring cell carcinoma 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. Signet-ring cell carcinoma typically occurs in the stomach, but can arise in almost every organ, including the large intestine, lung and breast, and can present as distant metastases. A typical example is the Krukenberg tumor, which is a metastatic signet-ring cell carcinoma that occurs in the ovary. Because this cancer has a morphological identity irrespective of the primary site of origin or metastatic status, it is difficult to determine the primary site. It was earlier confirmed that expression of Reg IV is limited to cancers of the stomach, colon and pancreas, whereas lung cancers and breast cancers do not express Reg IV. The immunohistochemical characteristics of signet-ring cell carcinoma were then analyzed from various organs including the stomach, colorectum, breast and lung, using antibodies against Reg IV and known tissue markers such as MUC2, MUC5AC, cytokeratin (CK) 7, CK20, caudal-related homeobox gene 2 (CDX2), thyroid transcription factor-1 (TTF-1), mammaglobin, gross cystic disease fluid protein-15 (GCDFP15), and estrogen receptor (ER).[36]

All gastric and colorectal signet-ring cell carcinomas were positive for Reg IV expression (Fig. 3e), whereas none of them expressed TTF-1, mammaglobin, GCDFP15 or ER. MUC2, MUC5AC, CK7, CK20 and CDX2 were expressed in 50–80% of gastric signet-ring cell carcinomas, while MUC2, CK20 and CDX2 were expressed in >80%, and MUC5AC and CK7 in 38% and 12% of colorectal signet-ring cell carcinoma, respectively. In contrast, none of the pulmonary and breast signet-ring cell carcinomas expressed Reg IV. While most of the pulmonary signet-ring cell carcinomas expressed TTF-1, most of the breast signet-ring cell carcinomas showed cytoplasmic staining for GCDFP15 and nuclear staining of ER. As expected, TTF-1 was a good tissue marker for signet-ring cell carcinomas originating from the lung, and GCDFP15 and ER were good markers for signet-ring cell carcinomas of