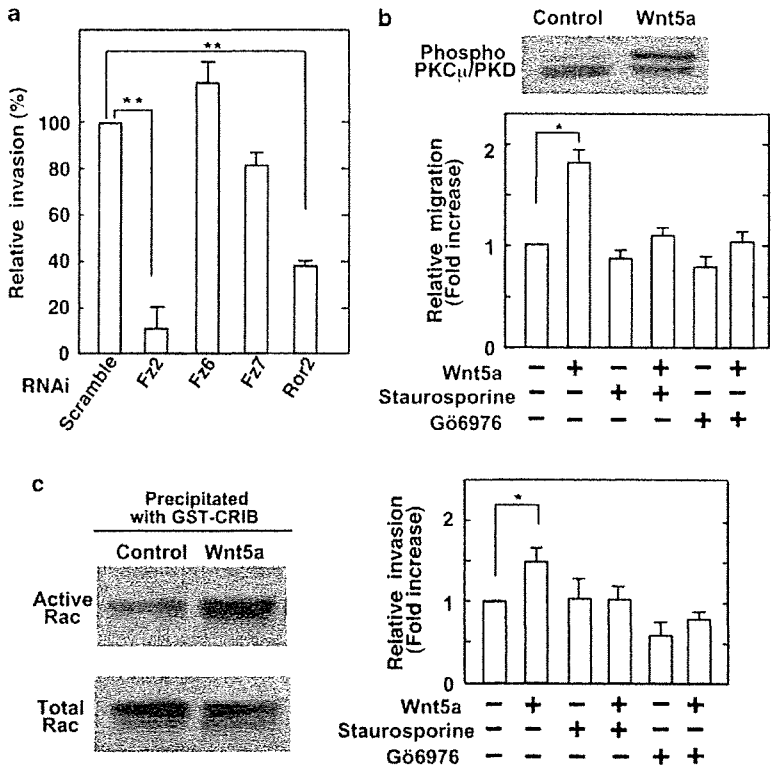


Figure 4 Wnt5a stimulates cell migration and invasion by activating protein kinase C (PKC). (a) DU145 cells transfected with *scrambled*, *Fz2*, *Fz6*, *Fz7* or *Ror2* siRNA were placed in Matrigel-coated transwell chambers for an invasion assay. In three independent fields, 37 ± 7 transfectants with *scrambled* siRNA invaded. Relative invasion activities were expressed as fold increases compared with the transfectants with *scrambled* siRNA. The results shown are means ± s.e. from three independent experiments. $^{**}P<0.01$. (b) Top panel, DU145 cells were stimulated with 300 ng/ml Wnt5a for 30 min, then the cell lysates were probed with anti-phospho protein kinase D (PKD)/PKCμ antibody. Middle panel, after DU145 cells were treated with 1.25 nM staurosporine or 100 nM Gö6976 for 2 h, the cells were subjected to the transwell migration assay in the presence or absence of 200 ng/ml Wnt5a. Relative migration activities were expressed as fold increases compared with that of cells in the absence of Wnt5a and without staurosporine. Bottom panel, after DU145 cells were treated with 2.5 nM staurosporine or 100 nM Gö6976 for 2 h, the cells were subjected to the Matrigel invasion assay in the presence or absence of 600 ng/ml Wnt5a. $^{*}P<0.05$. (c) DU145 cells were treated with 50 ng/ml Wnt5a for 1 h, and then cells were lyzed and probed with anti-Rac1 antibody. The same lysates were incubated with glutathione *S*-transferase (GST)-Cdc42/Rac-interacting binding domain (CRIB) immobilized on glutathione-sepharose to examine the activation of Rac. The total lysates and precipitates were probed with anti-Rac1 antibody. The results shown are representative of three independent experiments.

site (Doyle *et al.*, 1997; Wu *et al.*, 2006). In addition, knockdown of Fz2 and Ror2 reduced Wnt5a-dependent invasion and increment in *MMP-1* mRNA. From these results, it is suggested that the binding of Wnt5a to Fz2 and/or Ror2 stimulates the expression of MMP-1 by the recruitment of JunD to the AP-1 binding site of the promoter region of the *MMP-1* gene through the activation of PKC and JNK.

As shown in other cells (Kurayoshi *et al.*, 2006, 2007), Wnt5a activated Rac, which stimulates cell migration, in DU145 cells. However, the Wnt5a-Rac pathway was not involved in the expression of MMP-1. Therefore, it is also possible that Wnt5a activates Rac to stimulate cell migration independently of transcription and that this pathway cooperates with the Wnt5a/PKC pathway to stimulate cell invasion.

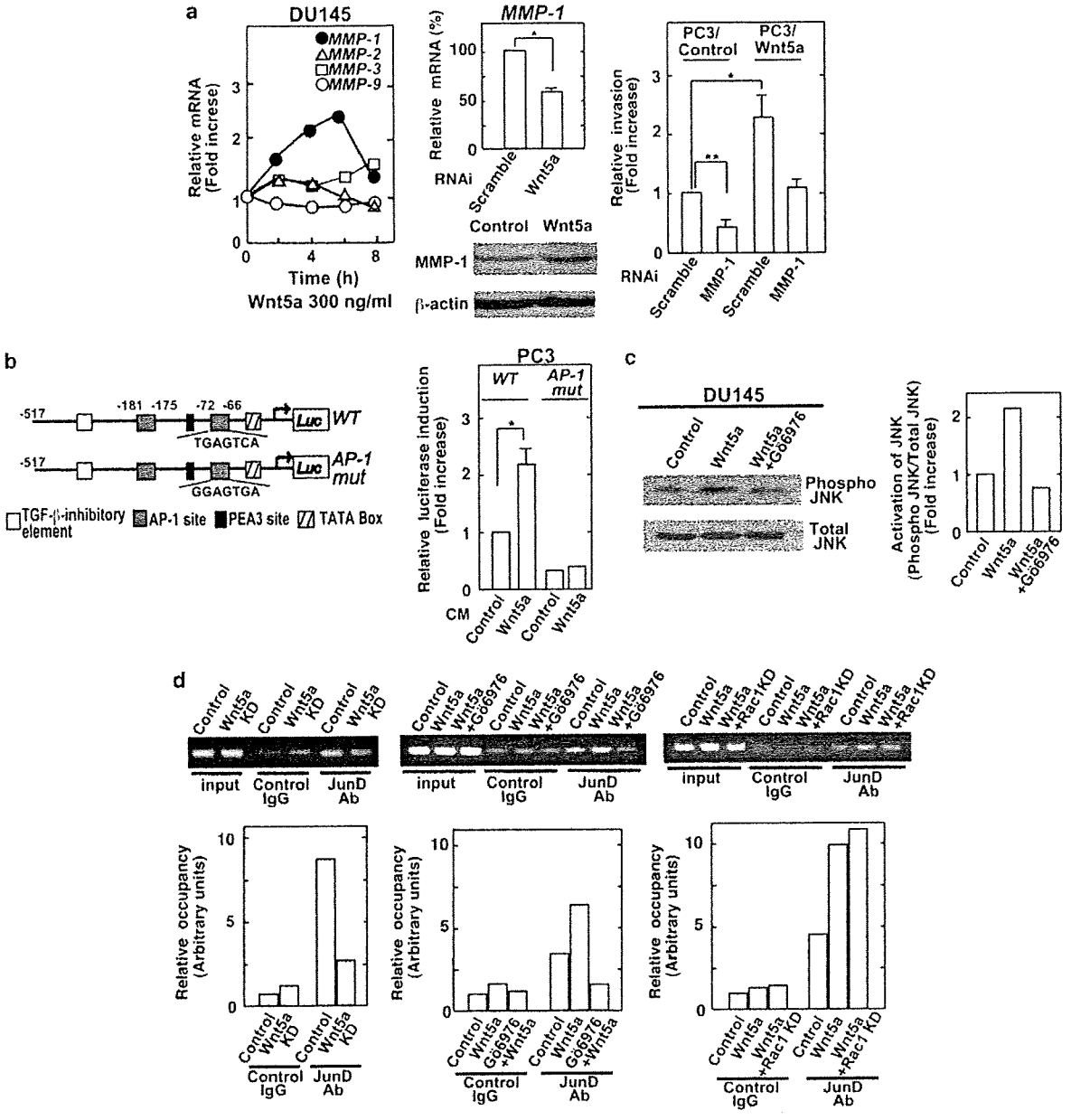
Although evidence has been accumulated that Wnt5a is expressed in various cancers (Weeraratna *et al.*, 2002; Veeman *et al.*, 2003; Huang *et al.*, 2005; Kurayoshi *et al.*, 2006; Pukrop *et al.*, 2006; Kikuchi and Yamamoto, 2008; Yamamoto *et al.*, 2009), how Wnt5a is upregulated in cancer cells has not been determined. It has been shown that Wnt5a is upregulated at the transcriptional level in PCa by hypomethylation in the 5′-untranslated region and that three CpG sites were consistently methylated in normal tissues but not in primary PCa (Wang *et al.*, 2007). It was also reported that membrane type 1-MMP is upregulated in PCa species and that membrane type 1-MMP-induced phenotypic changes are dependent on the expression of Wnt5a (Cao *et al.*, 2008). It is intriguing to speculate that Wnt5a induced by membrane type 1-MMP upregulates MMP-1 and these three molecules work cooperatively to stimulate cell migration and invasion in PCa cells. Various alterations, including gene amplification, genetic mutations, transcriptional activation and

epigenetic alterations, could upregulate Wnt5a expression in PCa. Further studies will be necessary to understand the functions of Wnt5a and the pathological significance of the abnormal expression of Wnt5a in cancer cells.

Materials and methods

Materials and chemicals

The *MMP-1* promoter-*luciferase* constructs and pGEX-αPAK-CRIB were provided by Dr I Clark (University of East Anglia, Norwich, UK) (Hall *et al.*, 2003) and Dr K Kaibuchi (Nagoya University, Nagoya, Japan), respectively. DU145, LNCap and PC3 cells were obtained from American Type Culture Collection (Manassas, VA, USA) and were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum. PC3 cells stably expressing mouse Wnt5a were generated by selection with 200 μg/ml G418. Wnt5a was purified to homogeneity, and an anti-Wnt5a antibody was generated as described previously (Kurayoshi *et al.*, 2006, 2007). sFRP2 CM was prepared from culture medium of HEK293T cells stably expressing sFRP2 as described previously (Kurayoshi *et al.*, 2006). Control CM and Wnt5a CM were prepared as described previously (Kurayoshi *et al.*, 2007).



Immunohistochemistry

Formalin-fixed and paraffin-embedded samples were sectioned, deparaffinized and stained with hematoxylin and eosin to ensure that the sectioned block contained tumor cells. Adjacent sections were then stained immunohistochemically. For immunostaining of Wnt5a, a Dako CSA Kit (Dako, Carpinteria, CA, USA) was used according to the manufacturer's recommendation. Sections were pretreated in a microwave oven in citrate buffer for 30 min to retrieve antigenicity. After peroxidase activity was blocked with 3% H_2O_2-methanol for 10 min, the sections were incubated with normal goat serum (Dako) for 20 min to block nonspecific antibody binding sites. The anti-Wnt5a antibody was incubated with tissue samples for 15 min at room temperature and detected by incubating for 15 min with biotinylated goat anti-rabbit immunoglobulins, and the signal was amplified and visualized using the substrate-chromogen solution. The sections were counterstained with 0.1% hematoxylin. The specificity of the anti-Wnt5a antibody has been characterized previously (Kurayoshi *et al.*, 2006, 2007).

For immunostaining of β-catenin, a Dako LSAB Kit (Dako) was used in accordance with the manufacturer's recommendations. After blocking nonspecific antibody binding sites, the samples were incubated with mouse monoclonal anti-β-catenin (1:20; BD Bioscience, San Jose, CA, USA), and followed by incubation with biotinylated anti-mouse 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. The staining of Wnt5a and cytosomal or nuclear β-catenin was classified according to the percentage of stained cancer cells in the tumor region. When more than 50% of cancer cells were stained, the immunostaining was considered positive.

Tissue samples

Ninety-eight primary tumors were collected from patients diagnosed with PCa who underwent surgery during the period 2000 through 2002 at the Department of Urology, Hiroshima University Hospital (Hiroshima, Japan). Identifying information for all samples was removed before analysis for strict privacy protection. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government, and the study was approved by the Ethical Committee for Human Genome Research of Hiroshima University (Hiroshima, Japan).

All patients were treated by radical prostatectomy and bilateral lymphadenectomy for clinically localized PCa and were confirmed to be node negative by pathological examination. None of the patients were treated preoperatively with hormonal or radiation therapy, and none had secondary cancer. All 98 specimens were archival, formalin-fixed and paraffin-embedded tissues. Tumor staging was performed according to the TNM classification system (Sobin and Wittekind, 2002). After prostatectomy, the serum PSA level was measured by E-test Tosoh II Assay (Tosoh, Tokyo, Japan). Patients were followed up by PSA measurement monthly during the first 6 months after prostatectomy and then every 3 months thereafter. Biochemical relapse was defined as a PSA level of 0.2 ng/ml or greater.

Statistical methods

Correlations between clinicopathological parameters and Wnt5a or β-catenin positivity were analyzed by Fisher's exact test. Kaplan–Meier curves were constructed, and differences between relapse-free survival curves were tested for statistical significance by log-rank test (Mantel, 1966). Cox proportional hazards multivariate model was used to examine the association of clinical and pathological factors and the expression of Wnt5a or β-catenin with relapse-free survival. Statistical analyses for Figures 3–5 were carried out using Student's *t*-test. A *P*-value of less than 0.05 was considered statistically significant.

Cell migration and invasion assays

To measure the cell migration activity, we performed transwell assays using a modified Boyden chamber (tissue culture treated, 6.5-mm-diameter, 10-μm-thickness, 8-μm-pores; Transwell, Costar, Cambridge, MA, USA) as described previously (Kobayashi *et al.*, 2006). The lower surface of the filters was coated with 10 μg/ml fibronectin for LNCap cells and 10 μg/ml type I collagen for DU145 cells. DU145 and LNCap cells (2.5×10^4 cells in 100 μl) suspended in serum-free RPMI-1640 medium containing 0.1% bovine serum albumin were applied to the upper chamber. The same medium was

Figure 5 Wnt5a induces the expression of matrix metalloproteinase-1 (MMP-1). (**a**) Left panel, DU145 cells were treated with 300 ng/ml Wnt5a for the indicated periods of time, and then *MMP-1*, *MMP-2*, *MMP-3* and *MMP-9* mRNA levels were quantified. Middle upper panel, *MMP-1* mRNA levels in DU145 cells transfected with *scrambled* or *Wnt5a* siRNA were quantified. The results shown are means ± s.e. from three independent experiments. $*P<0.05$. Middle lower panel, DU145 cells were stimulated with 300 ng/ml Wnt5a for 10 h, then the cell lysates were probed with anti-MMP-1 antibody. β-Actin is a loading control. Right panel, PC3/control and PC3/Wnt5a cells were transfected transiently with *scrambled* or *MMP-1* siRNA, and these cells were subjected to the Matrigel invasion assay. Relative invasion activities were expressed as fold increases compared with the invaded cell numbers in PC3/control cells transfected with *scrambled* siRNA. The results shown are means ± s.e. from three independent experiments. $*P<0.05$; $**P<0.01$. (**b**) Left panel, the *MMP-1-luciferase* constructs used in this study. Right panel, after PC3 cells were transfected with pGL3/*MMP-1 −517/+60-Luc* with or without mutations in the proximal activator protein-1 (AP-1) site (*AP-1 mut* or *WT*, respectively), the cells were stimulated with control or Wnt5a conditioned medium (CM) for 10 h. Luciferase activities were expressed as fold increases compared with that of *WT* transfectants treated with control CM. The results shown are means ± s.e. from three independent experiments. $*P<0.05$. WT, wild type. (**c**) After the treatment of Gö6976 for 2 h, DU145 cells were stimulated with 450 ng/ml Wnt5a for 1 h. The cell lysates were probed with anti-phospho-c-jun N-terminal kinase (JNK) antibody. Right panel, the activity of JNK was calculated by dividing the band intensity of phosphorylated JNK by that of total JNK and expressed as fold increases compared with control DU145 cells. The results shown are representative of three independent experiments. (**d**) Left panel, chromatins from DU145/control and DU145/Wnt5aKD cells were immunoprecipitated with anti-JunD antibody or control IgG. KD, knockdown. Middle panel, after DU145 cells were stimulated with 450 ng/ml Wnt5a for 1 h in the presence or absence of 100 nM Gö6976, chromatins were collected and were immunoprecipitated with anti-JunD antibody or control IgG. Right panel, after DU145 cells were transfected with *scrambled* or *Rac1* siRNA, the cells were stimulated with 450 ng/ml Wnt5a for 1 h. Chromatins from each cell were immunoprecipitated with anti-JunD antibody or control IgG. The immunoprecipitated samples were analyzed by real-time PCR for the *MMP-1* promoter region containing two AP-1 sites. The relative amounts of DNA fragments containing AP-1 sites immunoprecipitated with anti-JunD antibody were expressed as arbitrary units compared with that with control IgG in control DU145 cells. The results shown are representative of three independent experiments.

added to the lower chamber. After the cells were incubated at 37 °C for 4–8 h, the number of cells that migrated to the lower side of the upper chamber was counted. Relative cell migration was expressed as a percentage of migrated cells with siRNA treatment compared to those without treatment. The invasive potentials of DU145 and PC3 cells were analyzed using a Matrigel-coated modified Boyden chamber (Becton, Dickinson and Company, Bedford, MA, USA). RPMI-1640 medium containing 10% fetal bovine serum was added to the lower chamber. The incubations of DU145 and PC3 cells were continued for 24 and 4 h, respectively.

To carry out the wound-healing assay, we plated the cells onto fibronectin-coated coverslips. The monolayer of DU145 cells was then scratched manually with a plastic pipette tip, and after being washed with PBS, the wounded monolayers of cells were allowed to heal for 18–24 h in RPMI-1640 medium containing 10% fetal bovine serum. The length of the wounds was measured and expressed as a percentage of the initial length at zero time (Kobayashi *et al.*, 2006). When necessary, the anti-Wnt5a antibody (10 μg/ml) or CM containing sFRP2 was added to the medium.

Chromatin immunoprecipitation assay

Cells (2×10^6) were cross-linked with 1% formaldehyde for 10 min at room temperature. The cell pellets were lyzed with SDS lysis buffer and sonicated to shear DNA to a size range between 200 and 1000 bp. Sheared chromatin samples were diluted in chromatin immunoprecipitation buffer and incubated for 12 h at 4 °C with 6 μg of anti-JunD antibody (sc-74; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or negative control IgG (Diagenode, Liège, Belgium). Immunocomplexes were collected, and the DNA fragments were purified using DNA purifying slurry (Diagenode). After incubation at 55 °C for 30 min to reverse protein/DNA cross-links, the purified DNA was used as a template for PCR. Forward and reverse primers were as follows: fragment containing AP-1 sites of *MMP-1* promoter, 5′-TGTCTCCTTCGCACACATCT-3′ and 5′-TGCATACTGGCCTTTGTCTT-3′.

Reporter gene assay

PC3 cells were transfected with pGL3/*MMP-1* $-517/+60$-*Luc* and pME18S/LacZ. At 24 h after transfection, the cells were cultured in serum-free medium for 24 h and stimulated with Wnt5a CM for further 10 h, and then the luciferase activities were measured with PicaGene reagent (Toyo Ink, Tokyo, Japan). β-Galactosidase activities were determined to normalize the transfection efficiency.

Others

Rac activity was assayed using glutathione *S*-transferase fusion Cdc42/Rac-interacting binding domain as described previously (Kurayoshi *et al.*, 2006).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Original Article

Immunostaining of gastric cancer with neuroendocrine differentiation: Reg IV-positive neuroendocrine cells are associated with gastrin, serotonin, pancreatic polypeptide and somatostatin

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We previously reported that Reg IV is associated with neuroendocrine (NE) differentiation in gastric cancers. The aim was to examine which NE hormone products are related to Reg IV-positive NE cells and their roles in gastric cancers. In the present study, we performed immunohistochemical analysis in a tissue microarray (TMA) of a consecutive series of 630 cases with ten different antibodies, including chromogranin A, synaptophysin and neural cell adhesion molecule (NCAM) as NE differentiation markers, and gastrin, serotonin, calcitonin, gastrin-releasing peptide (GRP), pancreatic polypeptide (PP), somatostatin and glucagon as NE hormones. In 630 cases, we identified 205 (33%) with NE differentiation and 147 (23%) positive for Reg IV. Reg IV-positive cases showed NE differentiation more frequently than Reg IV-negative cases ($P < 0.0001$). In 205 cases with NE differentiation, Reg IV-positive cases expressed serotonin ($P = 0.0032$) and somatostatin ($P = 0.036$) more frequently than Reg IV-negative cases. Double immunofluorescence staining revealed co-expression of Reg IV with gastrin, serotonin and PP. These results indicate that Reg IV might be a mediating factor of several NE hormones.

Key words: gastric cancer, gastrin, neuroendocrine differentiation, Reg IV, serotonin

The presence of neuroendocrine (NE) differentiation in gastric carcinoma has been relatively well studied, with occurrences varying from 19% to 53% in the literature reported.[1,2] Neuroendocrine cells are found interspersed among adenocarcinoma cells in typical gastric cancers,[3,4] which must be distinguished from NE carcinomas with highly malignant biological behavior and extremely poor prognosis.[5] The clinical significance of NE differentiation in gastric cancer in general is still controversial, with reports of a better[6] and also a poorer associated prognosis.[7] On the other hand, NE neoplasms are varied in their biological behavior, depending on their cell type, and can produce different NE hormones causing distinct clinical endocrine syndromes.[7] Compared with NE carcinomas, little investigation has been carried out on the direct relationship between NE cells and their NE hormone products in non-NE cancers, especially in common gastric cancers.[6,8,9]

We previously performed serial analysis of gene expression (SAGE) of primary gastric cancers[10] and identified several gastric cancer-related genes[11] and useful diagnostic markers.[12] Of these genes, *Regenerating islet-derived family, member 4* (*REG4*, which encodes Reg IV) is a candidate gene for cancer-specific expression.[11] *REG4* is a member of the REG gene family, which includes three other genes, and was originally identified by high-throughput sequence analysis of a large inflammatory bowel disease cDNA library.[13] By quantitative reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemical analysis, overexpression of Reg IV was detected in 30–50% of gastric cancers.[10] Reg IV is also expressed in colorectal cancer,[14] pancreatic cancer,[15] prostate cancer,[16] and adenoid cystic carcinoma.[17] Immunohistochemically, there are two Reg IV staining patterns; mucin-like staining and perinuclear staining.[18] Mucin-like staining, observed in goblet cells and goblet cell-like vesicles of tumor cells, is associated with MUC2 (a marker of

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Table 1 Antibodies used in the current study

Antigen	Clone	Dilution	Pretreatment	Source
Reg IV	Polyclonal	1:50	MW	†
Chromogranin A	Polyclonal	1:50	MW	Novocastra, Newcastle on Tyne, UK
Synaptophysin	Polyclonal	1:50	MW	DAKO, Carpinteria, CA, USA
NCAM	1B6	1:50	MW	Novocastra, Newcastle on Tyne, UK
Gastrin	Polyclonal	1:50	MW	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Serotonin	5HT-H209	1:50	MW	DAKO, Carpinteria, CA, USA
Calcitonin	Polyclonal	1:50	MW	Novocastra, Newcastle on Tyne, UK
GRP	H-027-07	1:50	MW	Phoenix pharmaceuticals, Burlingame, CA, USA
PP	H-054-02	1:50	MW	Phoenix pharmaceuticals, Belmont, CA, USA
Somatostatin	Polyclonal	1:50	MW	DAKO, Carpinteria, CA, USA
Glucagon	Polyclonal	Diluted	MW	Nichirei, Tokyo, Japan

†Rabbit polyclonal anti-Reg IV antibody was raised in our laboratory.
GRP, gastrin-releasing peptide; NCAM, neural cell adhesion molecule; PP, pancreatic polypeptide; MW, microwaving (500W) in citrate buffer (pH 6.0) for 15 min.

goblet cells) positivity. Perinuclear staining is detected in cells with NE differentiation. However, it remains unclear which NE hormone products are related to Reg IV-positive NE cells.

In the present study, to characterize Reg IV-positive NE cells, immunohistochemical examination was carried out in a tissue microarray (TMA) of a consecutive series of 630 gastric cancers with ten different antibodies, including chromogranin A, synaptophysin and neural cell adhesion molecule (NCAM) as NE differentiation markers, and gastrin, serotonin, calcitonin, gastrin-releasing peptide (GRP), pancreatic polypeptide (PP), somatostatin and glucagon as NE hormones.

MATERIALS AND METHODS

Tissue samples and TMA construction

The surgical pathology files of the Hiroshima University Hospital, Japan, and its affiliated hospitals were used to randomly select 630 cases of gastric cancer. Surgically resected specimens were routinely fixed in 10% buffered formalin and examined macroscopically. Tumor staging was performed according to the Union Internationale Contre le Cancer (UICC) system.[19] Histological classification was carried out according to the Lauren classification system.[20] There were 112 Tis, 155 T1, 208 T2, 123 T3, and 32 T4 in these 630 cases. Nodal metastasis was present in 285 patients (45%). Tumor staging revealed 112 stage 0, 227 stage I, 113 stage II, 113 stage III, and 65 stage IV. Gastric cancers were histologically classified as 357 intestinal type and 273 diffuse type cancers. In accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government, tissue specimens were collected and used after approval from the Ethical Review Committee of the Hiroshima University School of Medicine and from the ethical review committees of collaborating organizations.

The two most representative tumor areas to be sampled for the TMAs were carefully selected and marked on the hematoxylin & eosin (HE)-stained slide in each case. Two superficial areas in mucosal gastric cancers, and one superficial area and one deep area in gastric cancers that had invaded punched out and transferred to a recipient block with a maximum of 48 cores using a Tissue Microarrayer (AZUMAYA KIN-1, Tokyo, Japan). Five-μm-thick sections were cut from the recipient block and transferred to glass slides. HE staining was performed on TMA for confirmation of the tumor tissue. Each tissue-array block contained 21 cases of gastric cancer and four cases of non-neoplastic stomach samples.

Immunohistochemistry

A Dako Envision Kit (DAKO, Carpinteria, CA, USA) was used for immunohistochemical analysis of all markers except gastrin. In brief, sections were pretreated by microwaving (500W) in citrate buffer (pH 6.0) for 15 min to retrieve antigenicity. After endogenous peroxidase activity was blocked with 3% H_2O_2-methanol for 10 min, sections were incubated with normal goat serum (DAKO) for 20 min to block non-specific antibody binding sites. Sections were then incubated with the following primary antibodies (Table 1): anti-Reg IV, anti-chromogranin A, anti-synaptophysin, anti-NCAM, anti-gastrin, anti-serotonin, anti-calcitonin, anti-GRP, anti-PP, anti-somatostatin, and anti-glucagon. Suppliers and working dilutions are noted in Table 1. Rabbit polyclonal anti-Reg IV antibody was raised in our laboratory.[18] The specificity of the Reg IV antibody has been characterized in detail.[18] Sections were incubated with primary antibody for 1 h at 25°C, followed by incubations with peroxidase-labeled anti-rabbit or mouse IgG for 60 min. For immunostaining of gastrin, peroxidase-conjugated anti-goat IgG was used as the secondary antibody. Staining was completed with a 10-minute incubation

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with the substrate-chromogen solution. The sections were counterstained with 0.1% hematoxylin. Appropriate positive and negative control samples were used.

Double-immunofluorescence staining was performed as described previously.[21] Alexa Fluor 546-conjugated anti-goat IgG (Molecular Probes, Eugene, OR, USA) and Alexa Fluor 488-conjugated anti-rabbit IgG (Molecular Probes) or Alexa Fluor 546-conjugated anti-mouse IgG (Molecular Probes) and Alexa Fluor 488-conjugated anti-rabbit IgG (Molecular Probes) were used as secondary antibodies.

Evaluation of positive cases and cutoff-point thresholds

Immunostaining was evaluated independently by two investigators (KS, NO), and when the evaluations differed, a decision was made by consensus while investigators reviewed the specimen with a multihead microscope. Neoplastic tissue was evaluated semiquantitatively at magnifications of ×100 and ×400. Cytoplasmic immunoreactivity for Reg IV, chromogranin A, synaptophysin, gastrin, serotonin, calcitonin, GRP, PP, somatostatin, and glucagon; and membranous reactivity for NCAM were assessed.

For the TMAs, staining was considered positive if any tumor cells were stained appropriately. The percentage of reactive cells necessary for a positive result reflects the viewpoint and opinion of the authors. There can be significant methodologic differences between studies and we are aware of the potential effect of these differences on a study's results. The aim of the present study was to analyze the presence or absence of various NE markers in gastric cancers. Therefore, the cutoff-point for antibody reactivity necessary to define a result as positive was staining of any (>0%) cells in the TMAs.

Statistical methods

Associations between clinicopathologic variables and immunostaining for neuroendocrine markers were analyzed by Fisher's exact test. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

Association between various NE markers and Reg IV in non-neoplastic gastric mucosa

In non-neoplastic gastric mucosa, all NE hormones examined, except GRP, were detected in both intestinal metaplasia and non-metaplastic gastric mucosa, whereas GRP was expressed only in non-metaplastic gastric mucosa. In intestinal metaplasia, goblet cells showed Reg IV expression in goblet cell vesicles (Fig. 1a). In addition, NE cells at the base of intestinal metaplasia displayed Reg IV staining in the perinuclear region (Fig. 1a). As reported previously, cells with Reg IV staining of the perinuclear region are NE cells; however, all NE cells are not always positive for Reg IV. In non-metaplastic gastric mucosa, Reg IV staining was not observed. The distribution of Reg IV staining was compared with the distribution of NE hormone staining in serial sections. Some Reg IV-positive NE cells expressed gastrin, serotonin, and PP. Double immunofluorescence staining revealed co-expression of Reg IV with gastrin (Fig. 2a), serotonin (Fig. 2b), and PP (not shown). However, there were several NE cells that were positive for Reg IV but not for gastrin, serotonin, or PP, and vice versa. Co-expression of Reg IV with the other NE hormones was not observed (data not shown).

Expression of various NE markers in gastric cancers and their correlation with clinicopathologic parameters

Of the 630 gastric cancers, 147 (23%) were identified as Reg IV-positive. NE markers detected in the 630 cases included 76 (12%) cases with chromogranin A, 157 (25%) cases with synaptophysin, and 11 (2%) cases with NCAM. A total of 205 (33%) cases were found to have NE differentiation, and Reg IV-positive cases showed NE differentiation more frequently than Reg IV-negative cases ($P < 0.0001$). The incidence of gastric cancer with production of NE hormones was 4% for gastrin, 3% for serotonin, 2% for calcitonin, 9% for GRP, 5% for PP, 3% for somatostatin, and 1% for glucagon. We investigated the relation between the expression of various NE markers and their clinicopathologic parameters. As shown in Table 2, the expression of synaptophysin, gastrin, and GRP was associated with intestinal type according to Lauren classification ($P = 0.0414$, 0.0281, and 0.0208). Positive expression of Reg IV, chromogranin A, and gastrin were significantly more frequent in gastric cancers of Tis/T1 than those in T2/3/4 ($P = 0.0057$, 0.0351, and 0.024). Furthermore, gastrin-positive cases were significantly more frequent in stage 0/I cases than stage II/III/IV cases ($P = 0.0105$). In contrast, no correlation was found between other NE markers (NCAM, serotonin, calcitonin, PP, somatostatin, and glucagons) and clinicopathologic parameters.

We further analyzed association between Reg IV and various NE hormones in gastric cancers. Of 147 Reg IV-positive cases, 63 (43%) showed both mucin-like staining and perinuclear staining (Fig. 1b), whereas the remaining 84 (57%) displayed only mucin-like staining. Gastric cancers showing only perinuclear staining were not found. Therefore, of 630 gastric cancers, 63 cases showed perinuclear Reg IV

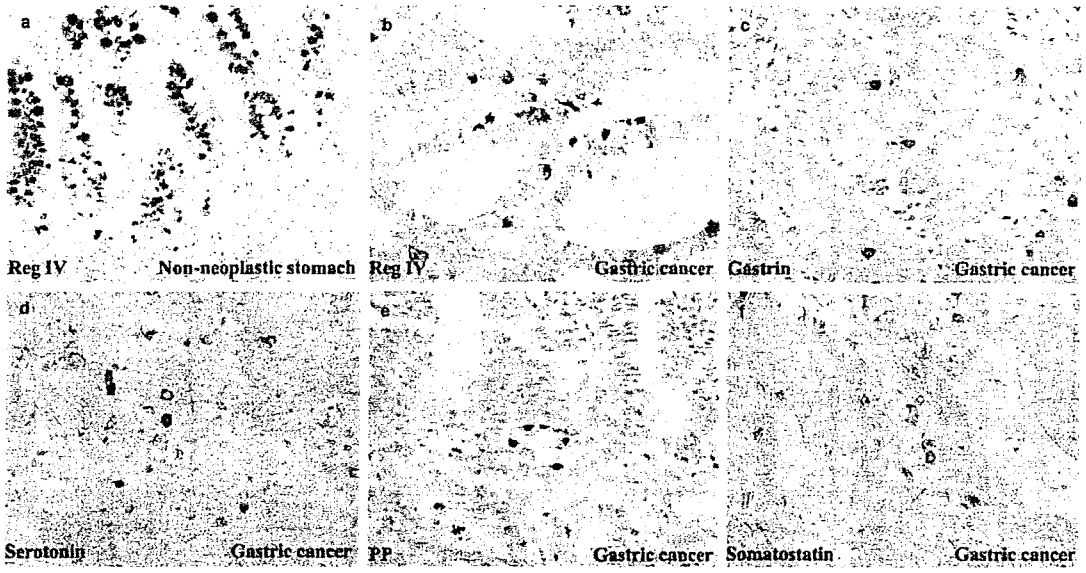

Figure 1 Immunohistochemical staining of Reg IV and neuroendocrine (NE) hormones. Reg IV showed positive staining in both goblet cell vesicles in goblet cells and the perinuclear region of neuroendocrine cells at the base of intestinal metaplasia (**a**). Reg IV showed positive staining in the perinuclear region of gastric cancer (**b**). Some gastric cancer cells showed production of NE hormones such as gastrin (**c**), serotonin (**d**), pancreatic polypeptide (PP) (**e**) and somatostatin (**f**).

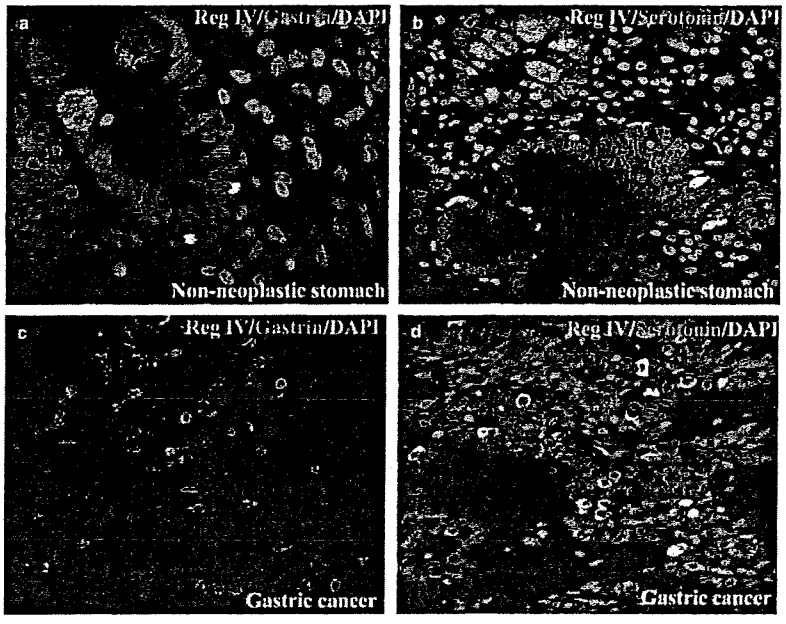

Figure 2 Double immunofluorescence staining revealed some non-neoplastic stomach cells with co-expression between Reg IV and gastrin (**a**), and Reg IV and serotonin (**b**). Some gastric cancer cells showed co-expression between Reg IV and gastrin (**c**), and Reg IV and serotonin (**d**). Cells were imaged with a fluorescence microscope as described in the Methods.

Table 2 Expression of various NE markers in gastric cancers and its correlation with clinicopathologic parameters

Histology† & TNM status	Reg IV	Chromogranin A	Synaptophysin	NCAM	Gastrin	Serotonin
Histology						
Intestinal type	85 (24%)	48 (13%)	100 (28%)	6 (2%)	21 (5%)	14 (3%)
Diffuse type	62 (23%)	28 (10%)	57 (21%)	5 (2%)	6 (2%)	7 (2%)
P-value	NS	NS	0.0414	NS	0.0281	NS
T grade						
Tis/T1	77 (29%)	41 (15%)	68 (25%)	4 (1%)	18 (6%)	10 (4%)
T2/T3/T4	70 (19%)	35 (10%)	89 (25%)	7 (2%)	9 (2%)	11 (3%)
P-value	0.0057	0.0351	NS	NS	0.0240	NS
N grade						
N0	87 (25%)	47 (14%)	87 (25%)	3 (0.9%)	18 (5%)	12 (3%)
N1/2/3	60 (21%)	29 (10%)	70 (25%)	8 (3%)	9 (3%)	9 (3%)
P-value	NS	NS	NS	NS	NS	NS
Staging						
Stage 0/I	89 (26%)	48 (14%)	88 (26%)	5 (1%)	21 (6%)	11 (3%)
Stage II/III/IV	58 (20%)	28 (10%)	69 (24%)	6 (2%)	6 (2%)	10 (3%)
P-value	NS	NS	NS	NS	0.0105	NS

Histology† & TNM status	Calcitonin	GRP	PP	Somatostatin	Glucagon
Histology					
Intestinal type	4 (1%)	39 (10%)	24 (6%)	11 (3%)	4 (1%)
Diffuse type	9 (3%)	15 (5%)	10 (3%)	5 (1%)	1 (0.4%)
P-value	NS	0.0208	NS	NS	NS
T grade					
Tis/T1	4 (1%)	28 (10%)	19 (7%)	7 (3%)	3 (1%)
T2/T3/T4	9 (2%)	26 (7%)	15 (4%)	9 (2%)	2 (0.6%)
P-value	NS	NS	NS	NS	NS
N grade					
N0	8 (2%)	31 (9%)	23 (7%)	9 (3%)	4 (1%)
N1/2/3	5 (2%)	23 (8%)	11 (4%)	7 (2%)	1 (0.4%)
P-value	NS	NS	NS	NS	NS
Staging					
Stage 0/I	6 (2%)	32 (9%)	22 (6%)	8 (2%)	4 (1%)
Stage II/III/IV	7 (2%)	22 (8%)	12 (4%)	8 (3%)	1 (0.3%)
P-value	NS	NS	NS	NS	NS

†Histologic classification was carried out according to the Lauren classification system. Tumor staging was performed according to the UICC system. GRP, gastrin-releasing peptide; NCAM, neural cell adhesion molecule; NE, neuroendocrine; NS, not significant; PP, pancreatic polypeptide.

staining. In contrast, 205 (33%) of 630 cases showed NE differentiation. All 63 gastric cancers with perinuclear Reg IV staining showed NE differentiation. To characterize Reg IV-positive NE cells, we focused on only gastric cancers showing perinuclear Reg IV staining. In 205 cases with NE differentiation, expression of serotonin was more frequently detected in Reg IV-positive cases (10/63, 16%) than in Reg IV-negative cases (5/142, 4%, $P = 0.0032$) (Table 3). In addition, expression of somatostatin was more frequently found in Reg IV-positive cases (8/63, 13%) than in Reg IV-negative cases (6/142, 4%, $P = 0.0360$) (Table 3). Expressions of other NE hormones were not correlated with Reg IV expression (Table 3). Next, the distribution of Reg IV staining was compared with the distribution of NE hormone staining in serial sections (Fig. 1c–f). Some Reg IV-positive cancer cells expressed gastrin, serotonin, and PP. Double immunofluorescence staining revealed co-expression of Reg IV with gastrin (Fig. 2c), serotonin (Fig. 2d), and PP. However, there were several cancer cells that were positive for Reg IV but not gastrin, serotonin, or PP, and vice versa. Co-expression of Reg IV with the other NE hormones was not observed (data not shown).

Table 3 Association between Reg IV and various NE hormones in 205 gastric cancers with NE differentiation

	Reg IV		
	Positive (63)	Negative (142)	Total 205 cases
Gastrin (+)	9 (14%)	12 (8%)	$P = 0.2181$
Serotonin (+)	10 (16%)	5 (4%)	$P = 0.0032$
Calcitonin (+)	4 (6%)	4 (3%)	$P = 0.2534$
GRP (+)	16 (25%)	25 (18%)	$P = 0.2557$
PP (+)	11 (17%)	16 (11%)	$P = 0.2642$
Somatostatin (+)	8 (13%)	6 (4%)	$P = 0.036$
Glucagon (+)	0 (0%)	3 (2%)	$P = 0.5543$

GRP, gastrin-releasing peptide; NE, neuroendocrine; PP, pancreatic polypeptide.

DISCUSSION

In the previous study, we revealed that expression of Reg IV is associated with both intestinal and neuroendocrine differentiation.[18] Perinuclear Reg IV staining is detected in cells with NE differentiation; however, it remains unclear which NE hormone products are related to Reg IV-positive NE cells. In the present study, some Reg IV-positive NE cells were also positive for gastrin, serotonin, and PP in both non-neoplastic gastric mucosa and gastric cancer. Reg IV has been identified as one of the genes that has an important role in intestinal epithelium development, homeostasis and function.[22] Because Reg IV-positive cases showed NE differentiation more frequently than Reg IV-negative cases, Reg IV may play a certain role in NE differentiation.

Both mucin-like staining and perinuclear staining of Reg IV are detected in intestinal metaplasia. It is known that Cdx2, a mammalian caudal-related intestinal transcription factor, is important for the maintenance of intestinal epithelial cells.[23,24] In addition, several lines of evidence have suggested that intestinal metaplasia of the stomach and gastric cancer with the intestinal mucin phenotype are associated with ectopic Cdx2 expression.[25–27] Because Cdx2 interacts with the MUC2 promoter and activates MUC2 transcription,[28] Cdx2 may regulate transcription of the REGIV gene. The NE cells at the base of intestinal metaplasia displayed Reg IV staining in the perinuclear region. A possible link between intestinal metaplasia of the stomach and NE cells has been observed in neurogenin-3 knockout mice.[29] In these mice, intestinal metaplasia occurs in the stomach, and glucagons-secreting A-cells, somatostatin-secreting D-cells, and gastrin-secreting G-cells are absent, whereas the number of serotonin-expressing enterochromaffin cells is decreased but present. These data suggest that NE cells in intestinal metaplasia may have origins different from those of other NE cells. Because Reg IV-positive NE cells are present in intestinal metaplasia, Reg IV may be involved in differentiation of serotonin-expressing enterochromaffin cells. In the previous report,[14] we revealed that both perinuclear and mucin-like staining of Reg IV were detected in non-neoplastic colorectal mucosa, and these perinuclear Reg IV-positive cells also express chromogranin A. In contrast, only mucin-like staining of Reg IV was observed in colorectal cancers, and not perinuclear Reg IV staining. Therefore, we speculated that the effects of Reg IV on NE cells differ a little in each organ.

Although the biological function of Reg IV is poorly understood, it has been reported that Reg IV is a potent activator of the epidermal growth factor receptor (EGFR)/Akt/activator protein-1 (AP-1) signaling pathway in colon cancer cells and increases expression of Bcl2, Bcl-xl and survivin associated with the inhibition of apoptosis.[30] We have also reported that forced expression of Reg IV induces phosphorylation of the EGFR and inhibits 5-fluorouracil-induced apoptosis in gastric cancer.[31] In the present study, some Reg IV-positive NE cells were also positive for gastrin in both non-neoplastic gastric mucosa and gastric cancer. Gastrin increases the expression of the EGFR ligands such as amphiregulin and EGF,[32] augments cell proliferation as well as angiogenesis and metastasis, and reduces apoptosis.[33] Because Reg IV is expressed in almost all EGFR-positive gastric cancers,[31] both Reg IV and gastrin may activate EGFR and may contribute to cancer cell growth. In addition, gastrin-positive cases expressed somatostatin and GRP more frequently than gastrin-negative cases (data not shown). This is consistent with the previous report that gastrin release is controlled by somatostatin and GRP in a negative and positive manner, respectively.[34]

In the current study, Reg IV-positive cases showed NE differentiation more frequently than Reg IV-negative cases, and showed an inverse correlation with the depth of tumor invasion. Neuroendocrine hormones regulate homeostasis by affecting cell proliferation, differentiation, apoptosis, and gene expression. The aberrant control of these biological processes is thought to play an important role in the establishment of neoplasia.[35,36] Further analysis is required to examine how the combination of Reg IV and NE hormones participates in the regulation of tumor initiation and development.

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Olfactomedin 4 (GW112, hGC-1) is an independent prognostic marker for survival in patients with colorectal cancer

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Abstract. Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths worldwide. We previously performed Serial Analysis of Gene Expression (SAGE) on four primary gastric cancer samples and identified several gastric cancer-specific genes. Of these genes, *olfactomedin 4* (*OLFM4*, also known as GW112 or hGC-1) is a candidate gene for cancer-specific expression. In the present study, we examined the expression and distribution of olfactomedin 4 in CRC by immunohistochemistry. Of the 176 CRC cases, 59 (34%) were positive for cytoplasmic staining of olfactomedin 4. Olfactomedin 4-positive CRC cases showed earlier T classification (P=0.0180), N classification (P=0.0149) and stage (P=0.0144) than olfactomedin 4-negative CRC cases. In the 176 CRC patients, those with olfactomedin 4-positive CRC had a better survival rate than patients with olfactomedin 4-negative CRC (P=0.0092). Multivariate analysis indicated that T classification, M classification and negative olfactomedin 4 expression were independent predictors of survival in patients with CRC. In addition to cytoplasmic staining of olfactomedin 4, stromal staining at the invasive front was observed. In total, 29 (16%) of the 176 CRC cases were positive for stromal olfactomedin 4; however, stromal olfactomedin 4 staining was not correlated with any clinicopathologic characteristic or with patient survival. These results indicate that olfactomedin 4 is a valuable marker for long-term survival in patients with CRC.

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related death worldwide. An assessment of prognosis based on features of the resected tumor would permit treating physicians to qualify the benefit of adjuvant chemotherapy to individual patients. Currently, anatomic and pathologic staging is still the most accurate predictor of patient outcome. It would be valuable to supplement standard clinical and pathologic staging using molecular markers to more precisely define the subset of patients at highest or lowest risk of relapse following CRC surgery. This would facilitate better selection of patients who would benefit most from adjuvant therapy. One of the most promising molecular markers is the presence of tumor microsatellite instability (1). We previously reported that expression of Reg IV and h-prune are prognostic makers for CRC (2,3); 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 CRC is important.

We previously performed Serial Analysis of Gene Expression (SAGE) on four primary gastric cancer samples (4) and identified several gastric cancer-specific genes (5). Of these genes, *olfactomedin 4* (*OLFM4*, also known as GW112 or hGC-1) is a candidate gene for cancer-specific expression, at least in patients with gastric cancer. *OLFM4* was originally cloned from human hematopoietic myeloid cells (6). Although *OLFM4* is predominantly expressed in bone marrow, the small intestine, colon and prostate (6), levels of expression are much lower in normal tissues than in gastric cancer tissues (5). Enhanced olfactomedin 4 expression has been reported in gastric cancer by Northern blot analysis (7) and by immunostaining (8). Our previous immunohistochemical analysis revealed that olfactomedin 4 is expressed in 56% of gastric cancer tissues (9). In addition, olfactomedin 4 is a secreted protein, and we showed that serum olfactomedin 4 represents a novel biomarker for gastric cancer (9). In CRC patients, preoperative serum levels of olfactomedin 4 were increased in a small number of samples, and the sensitivities of serum olfactomedin 4 at stage I-III were lower than those of CEA (9).

In addition to gastric cancer, *OLFM4* mRNA and olfactomedin 4 protein overexpression have been reported in CRC (10,11). Olfactomedin 4 inhibits apoptosis and may have significant roles in the development of cancer (7). It has been proposed that olfactomedin 4 can serve as a useful marker for stem cells in the human small intestine and colon (12). In

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Key words: olfactomedin 4, *OLFM4*, colorectal cancer, prognosis

contrast to these observations, immunohistochemical analysis has demonstrated that olfactomedin 4 down-regulation is found in late stage CRC cases and in CRC patients with shorter survival (13). The morphology and actin distribution of the HT-29 CRC cell line was altered by forced expression of olfactomedin 4. Forced expression of olfactomedin 4 did not change cell proliferation, but decreased cell adhesion and migration (13). Our previous immunohistochemical analysis in gastric cancer revealed that patients with olfactomedin 4-positive gastric cancer had a better survival rate than patients with olfactomedin 4-negative gastric cancer. These results suggest that olfactomedin 4 can inhibit tumor progression. Thus, the clinical significance of olfactomedin 4 expression in human cancers is controversial and still unclear.

Although immunohistochemical analysis of olfactomedin 4 has been performed in CRC (13), this study was performed using tissue microarray. Therefore, detailed expression and distribution of olfactomedin 4 in CRC has not yet been investigated. In the present study, we examined the expression and distribution of olfactomedin 4 in CRC by immunohistochemistry and the relationship between olfactomedin 4 staining and clinicopathologic characteristics.

Materials and methods

Tissue samples. In a retrospective study design, 176 primary tumors were collected from patients diagnosed with CRC who underwent surgery at Hiroshima University Hospital (Hiroshima, Japan). All patients underwent curative resection. Only patients without preoperative radiotherapy or chemotherapy were enrolled in the study. The patients were comprised of 105 men and 71 women. The mean age was 63 years (range, 29-89 years). Postoperative follow-up was scheduled every 1, 2 or 3 months during the first 2 years after surgery and every 6 months thereafter, unless more frequent follow-up was deemed necessary. Chest X-rays, chest computed tomography scans and serum chemistries were performed at every follow-up visit. Recurrence was evaluated from records at Hiroshima University Hospital. For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues. Histologic classification was based on the World Health Organization system. Tumor staging was performed according to the TNM stage grouping system (14). Since 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. From each patient, one or two representative tumor blocks, including the tumor center, invading front and tumor-associated non-neoplastic mucosa, were examined by immunohistochemistry. In cases of large, late-stage tumors, two different sections were examined to include representative areas of the tumor center as well as of the lateral and deep tumor invasive front. Olfactomedin 4 was detected immunohistochemically with a monoclonal antibody raised in our laboratory (9). The specificity of the anti-olfactomedin 4 antibody has been characterized in detail (9). A Dako Envision+ Mouse Peroxidase Detection System (Dako Cytomation, Carpinteria, CA, USA) was used for immunohistochemical analysis as described previously (9). In brief, antigen retrieval was carried out 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 antibody against olfactomedin 4 (1:50) for 1 h at room temperature, followed by incubations with Envision+ anti-mouse peroxidase for 1 h. Staining was completed with a 10-min incubation with the substrate-chromogen solution. Sections were counterstained with 0.1% hematoxylin. Negative controls were created by omission of the primary antibody.

Statistical methods. Correlations between clinicopathologic parameters and olfactomedin 4 expression were analyzed by the Chi-square 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 the log-rank test (15). The Cox proportional hazards multivariate model was used to examine the association of clinical and pathologic factors and the expression of olfactomedin 4 with survival. A P-value of <0.05 was considered statistically significant.

Results

Expression and distribution of olfactomedin 4 in CRC and peritumoral mucosa. We performed immunohistochemical analysis of olfactomedin 4 in 176 human CRC samples. In CRC tissue, olfactomedin 4 staining was frequently observed in well-differentiated (Fig. 1A) and moderately differentiated adenocarcinoma (Fig. 1B). In general, staining for 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 in olfactomedin 4 expression at the front of the invasion is observed in CRC (13); however, the tendency for loss of olfactomedin 4 expression at the invasive front was not observed. In our previous immunohistochemical analysis of gastric cancer (9), in addition to cytoplasmic staining, extracellular staining of olfactomedin 4 was observed. In CRC tissues, extracellular staining of olfactomedin 4 was also observed. Extracellular staining of olfactomedin 4 was focal, and in general, extracellular staining of olfactomedin 4 was observed at the invasive front (Fig. 1C). The immunoreactivity for olfactomedin 4 was irregular and fibrous around tumor cells scattered in the stroma (Fig. 1D).

We then focused on the peritumoral mucosa of CRC. Notably, strong and extensive olfactomedin 4 staining was detected, and all peritumoral mucosa samples in the 176 CRC cases were positive for olfactomedin 4 regardless of the olfactomedin 4 staining in tumor cells. Olfactomedin 4 staining decreased gradually, moving away from the CRC tissue. In the mucosa closest to the tumor tissue, almost all epithelial cells showed olfactomedin 4 staining (Fig. 1E). In contrast, in the mucosa distant from the tumor tissue, few epithelial cells

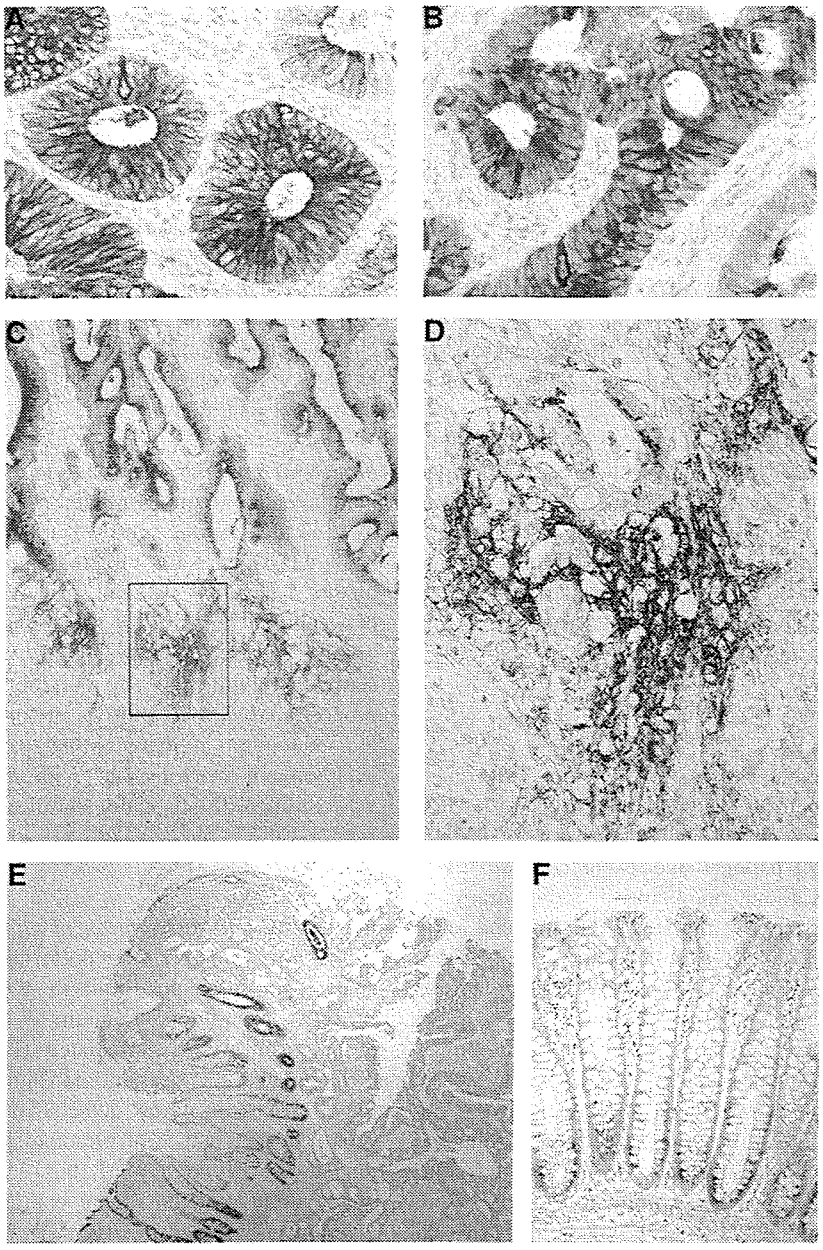

Figure 1. Immunohistochemical analysis of olfactomedin 4 in CRC and peritumoral mucosa. (A) Immunostaining of olfactomedin 4 in well-differentiated CRC. Staining of olfactomedin 4 was detected in the cytoplasm of tumor cells. Original magnification x400. (B) Immunostaining of olfactomedin 4 in moderately differentiated CRC. Staining of olfactomedin 4 was detected in the cytoplasm of tumor cells. Original magnification x400. (C) Immunostaining of olfactomedin 4 in CRC. Extracellular staining of olfactomedin 4 was observed at the invasive front. Original magnification x100. (D) High-magnification image of the field indicated by the box in C. The immunoreactivity for olfactomedin 4 was irregular and fibrous around tumor cells scattered in the stroma. Original magnification x400. (E) Immunostaining of olfactomedin 4 in the peritumoral mucosa of CRC. Almost all epithelial cells showed olfactomedin 4 staining. Original magnification x40. (F) Immunostaining of olfactomedin 4 in the mucosa distant from the tumor tissue. Few epithelial cells showed olfactomedin 4 staining. Original magnification x200.

showed olfactomedin 4 staining (Fig. 1F). Olfactomedin 4 was expressed in the basal crypt epithelium in the colon.

Relationship between olfactomedin 4 staining and clinicopathologic characteristics. The relationship of olfactomedin 4 staining with clinicopathologic characteristics was investigated (Table I). The level of olfactomedin 4 immunoreactivity was first evaluated in tumor cells. When >10% of tumor cells were stained, the immunostaining was considered positive for olfactomedin 4. In total, 59 (34%) of the 176 CRC cases were positive for olfactomedin 4. Olfactomedin 4-positive CRC cases showed earlier T classification (P=0.0180), N classification (P=0.0149) and stage (P=0.0144, all by the Chi-square test) than olfactomedin 4-negative CRC cases (Table I). Olfactomedin 4 staining was not correlated with age, gender, tumor location, M classification, or histologic classification. We also examined the relation between survival and olfactomedin 4 staining in CRC. In the 176

Table I. Correlation of olfactomedin 4 expression with clinicopathologic characteristics of 176 CRC cases.

	Olfactomedin 4 expression		
	Positive	Negative	P-value[a]
Age			0.9551
≤65	31 (33%)	62	
>65	28 (34%)	55	
Gender			0.7943
Male	36 (34%)	69	
Female	23 (32%)	48	
Tumor location			0.8075
Right/transverse	12 (35%)	22	
Left/sigmoid/rectum	47 (33%)	95	
T classification			0.0180
T1	14 (47%)	16	
T2	15 (39%)	23	
T3	24 (31%)	54	
T4	6 (20%)	24	
N classification			0.0149
N0	43 (41%)	63	
N1	16 (23%)	54	
M classification			0.6085
M0	53 (34%)	102	
M1	6 (29%)	15	
Stage			0.0144
I	26 (46%)	31	
II	16 (36%)	29	
III	11 (21%)	42	
IV	6 (29%)	15	
Histological classification			0.1973
Well/moderately	58 (35%)	110	
Poorly/mucinous	1 (13%)	7	

[a]Chi-square test.

CRC patients, those with olfactomedin 4-positive CRC had a better survival rate than patients with olfactomedin 4-negative CRC (P=0.0092, log-rank test) (Fig. 2A). It is well known that patients with CRC at stage I have a favorable rate of survival, whereas patients with CRC 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 CRC. Therefore, we analyzed the prognostic value of olfactomedin 4 in patients with stage II and III CRC. In stage II and III CRC patients (n=98), those with olfactomedin 4-positive CRC had a better survival rate than patients with olfactomedin 4-negative CRC (P=0.0347, log-rank test) (Fig. 2B). We then used Cox proportional hazards multivariate model to examine the association of clinicopathologic factors and expression of olfactomedin 4 with survival. Multivariate analysis indicated that T classification, M classification and olfactomedin 4 expression were independent predictors of survival in patients with CRC (Table II).

The level of olfactomedin 4 immunoreactivity was also evaluated in the tumor-associated stroma. Since extracellular staining of olfactomedin 4 at the invasive front was frequently observed, stromal olfactomedin 4 staining was considered positive when extracellular staining of olfactomedin 4 was stained at the invasive front. In total, 29 (16%) of the 176 CRC cases were positive for stromal olfactomedin 4. Stromal olfactomedin 4 staining was not correlated with age, gender, tumor location, T classification, N classification, M classification, stage, or histologic classification (data not shown). In the 176 CRC patients, survival rate was not statistically different between patients with stromal olfactomedin 4-positive CRC and those with stromal olfactomedin 4-negative CRC (data not shown).

Discussion

Previously, we performed SAGE on four primary gastric cancers (4) and identified several gastric cancer-specific genes (5). Of these genes, olfactomedin 4 is a candidate gene for cancer-specific expression. In the present study, we examined the expression and distribution of olfactomedin 4 in CRC by immunohistochemistry and the relationship between

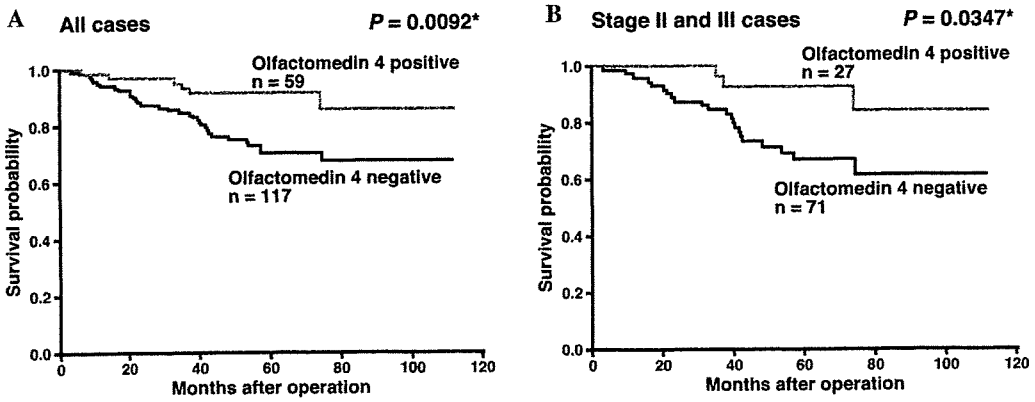

Figure 2. Survival of patients with CRC. (A) Kaplan-Meier curves of the CRC patients (all stages) with olfactomedin 4-negative or olfactomedin 4-positive tumors. (B) Kaplan-Meier curves of stage II and stage III CRC patients with olfactomedin 4-negative and olfactomedin 4-positive tumors. *Log-rank test.

Table II. Multivariate analysis of factors that influence survival of patients with CRC.

Factor	Hazard ratio	(95% CI)	Chi-square test	P-value[a]
Age			1.586	0.2078
≤65	1	(Reference)		
>65	1.511	(0.795-2.871)		
Gender			0.708	0.4001
Male	1	(Reference)		
Female	0.748	(0.381-1.470)		
Tumor location			0.368	0.5441
Right/transverse	1	(Reference)		
Left/sigmoid/rectum	0.745	(0.287-1.931)		
T classification			5.995	0.0143
T1/2	1	(Reference)		
T3/4	4.803	(1.367-16.869)		
N classification			3.488	0.0618
N0	1	(Reference)		
N1	7.327	(0.906-59.248)		
M classification			11.161	0.0008
M0	1	(Reference)		
M1	3.631	(1.704-7.737)		
Stage			1.032	0.3097
I/II	1	(Reference)		
III/IV	3.254	(0.334-31.713)		
Histologic classification			0.002	0.9632
Well/moderately differentiated	1	(Reference)		
Poorly differentiated /mucinous	0.936	(0.194-4.773)		
Olfactomedin 4 expression			4.486	0.0342
Positive	1	(Reference)		
Negative	2.725	(1.078-6.890)		

CI, Confidence interval. [a]Cox proportional hazards model.

olfactomedin 4 staining and clinicopathologic characteristics. Although few epithelial cells in colonic mucosa distant from the CRC tissue showed olfactomedin 4 staining, strong and extensive olfactomedin 4 staining was found in 34% cases of CRC, and olfactomedin 4-positive CRC cases showed earlier T classification, N classification and stage than olfactomedin 4-negative CRC cases. These results are consistent with results reported previously that olfactomedin 4 expression is up-regulated in early stage CRC and down-regulated in advanced stage CRC (13). In our previous study in gastric cancer, olfactomedin 4-positive cases were found frequently in early stage cases (9). Taken together, expression of olfactomedin 4 is an early event, and loss/reduction of olfactomedin 4 expression is a late event in gastrointestinal malignancies.

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 (16). 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 β-mediated cellular apoptosis, and transient expression of olfactomedin 4 promoted tumor growth in C57/BL/6 mice (7). Therefore, expression of olfactomedin 4 may contribute to carcinogenesis by resistance to apoptosis at least in early stage CRC. In contrast, forced expression of olfactomedin 4 in an HT-29 cell line decreased cell adhesion and migration (13). Therefore, it is possible that loss/reduction of olfactomedin 4 expression induces tumor cell invasion in late stage CRC cases.

In the present study, univariate and multivariate analyses revealed that negative expression of olfactomedin 4 is a prognostic indicator. Furthermore, negative expression of olfactomedin 4 correlated with a short survival rate in stage II and III CRC cases. Patients diagnosed with stage II or III CRC 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 olfactomedin 4 immunostaining is useful for identification of patients most likely to benefit from adjuvant treatment, association between

olfactomedin 4 staining and response to adjuvant therapies should be investigated.

In addition to cytoplasmic olfactomedin 4 staining, extracellular staining was also observed. Extracellular staining of olfactomedin 4 at the invasive front was frequently observed. Observation of the invasive front is important in the analysis of tumor cells, since it reflects the invasive potential of tumor cells. It has been reported that the expression of matrilysin in the invasive front is a promising biomarker predicting nodal metastasis of CRC (17). Overexpression of heparanase at the invasive front has been reported in gastric cancer and high expression of heparanase was a strong predictor of poor survival (18). These results indicate that the proteolytic degradation of the extracellular matrix by these molecules is one of the most important mechanisms in tumor progression, and the proteolytic degradation occurs at the invasive front. Although there was no correlation between stromal expression of olfactomedin 4 and clinicopathologic characteristics, stromal expression of olfactomedin 4 at the invasive front may partly contribute to the malignant behavior of CRC, such as local invasiveness.

Notably, extensive olfactomedin 4 staining was observed in the peritumoral mucosa of CRC, and olfactomedin 4 staining decreased gradually, moving away from the tumor tissue. It is well known that the peritumoral mucosa of CRC is often hyperplastic, and various growth factors, such as transforming growth factor-α and basic fibroblast growth factor, are increased in the peritumoral mucosa (19). Since expression of olfactomedin 4 in the crypt epithelium of inflamed colonic mucosa has been reported (20), olfactomedin 4 expression may be induced by growth factors and may function as an antiapoptotic factor in the peritumoral mucosa of CRC.

In summary, we showed that olfactomedin 4 is a valuable marker for long survival in patients with CRC. However, the significance of extracellular staining of olfactomedin 4 at the invasive front and extensive olfactomedin 4 staining in the peritumoral mucosa of CRC remains unclear. Since olfactomedin 4 is a secreted protein, identification of a cell surface receptor for olfactomedin 4 will further improve our understanding of the basic biology of olfactomedin 4.

Acknowledgements

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Serum olfactomedin 4 (GW112, hGC-1) in combination with Reg IV is a highly sensitive biomarker for gastric cancer patients

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Gastric cancer (GC) is 1 of the most common human cancers. Early detection remains the most promising approach to improving long-term survival of patients with GC. We previously performed Serial Analysis of Gene Expression (SAGE) on 4 primary GCs and identified several GC-specific genes including Reg IV. Of these genes, *olfactomedin 4* (*OLFM4*, also known as GW112 or hGC-1) is a candidate gene for cancer-specific expression. In this study, we examined the expression of olfactomedin 4 in human GC by immunohistochemistry. We also assessed serum olfactomedin 4 levels in GC patients by enzyme-linked immunosorbent assay. 94 (56%) of 167 GC cases were positive for olfactomedin 4 by immunostaining. Olfactomedin 4 staining was observed more frequently in stage I/II cases than in stage III/IV cases. The serum olfactomedin 4 concentration in presurgical GC patients (n = 123, mean ± SE, 36.3 ± 3.5 ng/mL) was significantly higher than that in healthy individuals (n = 76, 16.6 ± 1.6 ng/mL). In patients with stage I GC, the sensitivity of serum olfactomedin 4 (25%) and Reg IV (35%) was superior to that of CA19-9 (5%) or CEA (3%). Furthermore, in patients with stage I GC, the combination of olfactomedin 4 and Reg IV elevated the diagnostic sensitivity to 52%. These results suggest that serum olfactomedin 4 is a useful marker for GC and its measurement alone or in combination with Reg IV has utility in the early detection of GC.
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Key words: olfactomedin 4; OLFM4; Reg IV; serum tumor marker; gastric cancer

Gastric cancer (GC) is 1 of the most common human cancers. Early detection remains the most promising approach to improving long-term survival of patients with GC. Assessment of tumor markers in serum may be useful for detection of GC. There are 2 available tumor markers for GC, carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9). However, only 11 to 50% of patients with GC are positive for serum CEA before surgery, and 16–35% are positive for CA19-9.[1] Moreover, CEA and CA19-9 are not suitable for early screening because preoperative positivity for these markers depends on the tumor stage at the time of detection.[1] Therefore, there is an urgent need for new biomarkers for GC.

Better knowledge of changes in gene expression that occur during gastric carcinogenesis may lead to improvements in diagnosis, treatment, and prevention of GC. Genes encoding transmembrane/secretory proteins expressed specifically in cancers may be ideal diagnostic biomarkers.[2] Moreover, if the gene product functions in the neoplastic process, the gene is not just a biomarker but may also be a therapeutic target.[3] We previously performed Serial Analysis of Gene Expression (SAGE) on 4 primary GCs[4] and identified several GC-specific genes.[5] Of these genes, *regenerating islet-derived family, member 4* (*REG4*, which encodes Reg IV) is a candidate gene for cancer-specific expression. Reg IV is expressed in 30% of GCs and is associated with both the intestinal mucin phenotype and neuroendocrine differentiation.[6] Reg IV is a secreted protein, and we also showed that serum Reg IV represents a novel biomarker for GC.[7] In our previous study, the diagnostic sensitivity and specificity of serum Reg IV for detection of GC were 36 and 99%, respectively. In addition to Reg IV, *olfactomedin 4* (*OLFM4*, also known as GW112 or hGC-1) is a candidate gene for cancer-specific expression, at least in patients with GC.[5] *OLFM4* was originally cloned from human haematopoietic myeloid cells.[8] Although *OLFM4* is predominantly expressed in bone marrow, small intestine, colon and prostate,[8] the levels of expression are much lower in normal tissues than in GC tissues.[5] Enhanced olfactomedin 4 expression has been reported in GC by Northern blot analysis[9] and by immunostaining.[10] Because olfactomedin 4 is a secreted N-linked glycoprotein, olfactomedin 4 may serve as a specific serum biomarker for GC; however, the concentration of olfactomedin 4 in serum has not been investigated.

In addition to GC, *OLFM4* mRNA overexpression has been reported in colorectal cancer (CRC) as well as breast and lung cancer.[11] In contrast, immunohistochemical analysis has demonstrated that olfactomedin 4 down-regulation is found in late stage cases, and in patients with shorter survival of colon cancer.[12] The morphology and actin distribution of the HT-29 colon cancer cell line is altered by forced expression of olfactomedin 4. Forced expression of olfactomedin 4 does not change cell proliferation, but decreases cell adhesion and migration.[12] Therefore, olfactomedin 4 is involved in colon cancer adhesion and metastasis. 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 patient survival was not investigated in GC.

In this study, we examined the expression and distribution of olfactomedin 4 in human GC by immunohistochemistry, and the relationship between olfactomedin 4 staining and clinicopathologic characteristics. In addition, because olfactomedin 4 is expressed in intestinal metaplasia of the stomach, we investigated the association between olfactomedin 4 expression and the mucin phenotype in GC. We also assessed serum olfactomedin 4 levels in GC patients by an enzyme-linked immunosorbent assay (ELISA). Serum Reg IV, CA19-9 and CEA levels were also measured to investigate the potential diagnostic utility of olfactomedin 4 determination.

Material and methods

Cell line, expression vector and transfection

Four cell lines derived from human GC were used. Four GC cell lines of the MKN series (MKN-1, adenosquamous cell carci-

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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