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Serial analysis of gene expression of esophageal squamous cell carcinoma: *ADAMTS16* is upregulated in esophageal squamous cell carcinoma

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Esophageal squamous cell carcinoma (ESCC) is one of the most common malignancies worldwide. To identify potential diagnostic markers for ESCC and therapeutic targets for ESCC, we used Serial Analysis of Gene Expression (SAGE) on one ESCC sample. We obtained a total of 14 430 tags, including 5765 that were unique. By comparing SAGE tags from the ESCC sample with those from normal human squamous esophagus, we found several genes that were differentially expressed between ESCC and normal squamous esophagus. Among these, we focused on the ADAM metallopeptidase with thrombospondin type 1 motif, 16 (*ADAMTS16*) gene because quantitative RT-PCR analysis showed a high level of *ADAMTS16* expression in eight out of 20 ESCC samples (40%), but not in 15 kinds of normal tissues. Western blot analysis also showed upregulation of ADAMTS16 protein in ESCC tissues. Furthermore, ADAMTS16 protein was detected in culture media from the TE5 esophageal cancer cell line. Knockdown of ADAMTS16 in TE5 cells inhibited both cell growth and invasion ability. Our present SAGE data provide a list of genes potentially associated with ESCC. ADAMTS16 could be a novel diagnostic and therapeutic target for ESCC. (*Cancer Sci* 2010; 101: 1038–1044)

Human esophageal cancer occurs worldwide with a variable geographic distribution and ranks eighth in order of occurrence and sixth as a leading cause of cancer mortality, affecting men more than women.[1] There are two main forms, each with distinct etiologic and pathologic characteristics, esophageal squamous cell carcinoma (ESCC) and adenocarcinoma. ESCC is the most frequent subtype of esophageal cancer, although the incidence of adenocarcinoma in the USA and UK is increasing faster than other esophageal malignancies. Most ESCC is diagnosed at an advanced stage, and even superficial ESCC that appears to extend no further than the submucosa metastasizes to the lymph nodes in 50% of cases.[2] In spite of the use of modern surgical techniques combined with various treatment modalities, such as chemoradiotherapy (CRT), the overall 5-year survival rate of ESCC still remains at 40–60%.[3] Therefore, identification of new diagnostic markers for ESCC and new therapeutic targets for ESCC is important.

Better knowledge of changes in gene expression that occur during carcinogenesis might lead to improvements in diagnosis, treatment, and prevention of ESCC. Genes encoding transmembrane/secretory proteins expressed specifically in cancers may be ideal diagnostic biomarkers.[4] Moreover, if the gene product functions in the neoplastic process, the gene is not just a biomarker but might also be a therapeutic target.[5] To identify potential markers for early detection of ESCC and therapeutic targets for ESCC, comprehensive gene expression analysis could be useful. Studies on differential global gene expression profiling in ESCCs using cDNA and oligonucleotide arrays have been carried out in various populations.[6,7] Although many studies have been done on gene expression profiling of specific tumor types, and differentially expressed genes in these tumors have been reported, few of these studies have resulted in clinical applications. However, among the comprehensive methods used to analyze transcript expression levels, Serial Analysis of Gene Expression (SAGE) is a common approach.[8] We previously carried out SAGE on four primary gastric cancer tissues[9] and identified several gastric cancer-specific genes.[10] Of these genes, regenerating islet-derived family, member 4 (*REG4*, which encodes Reg IV) and olfactomedin 4 (*OLFM4*, also known as GW112 or hGC-1) are highly sensitive serum markers for gastric cancer.[11,12] However, SAGE analysis on ESCC tissue has been done in only one case.[13]

In the present study, we generated the SAGE library from one ESCC sample. By comparing SAGE tags from ESCC samples with those from normal human squamous esophagus (Gene Expression Omnibus accession number, GSM52501),[14] we found several genes and tags that were differentially expressed between ESCC and normal squamous esophagus. Among these, we focused on the ADAM metallopeptidase with thrombospondin type 1 motif, 16 (*ADAMTS16*) gene because it is frequently overexpressed in ESCC, and *ADAMTS16* expression is narrowly restricted among various normal tissues. In addition, the amino acid sequence of the ADAMTS16 protein suggests that it might be secreted. ADAMTS has been described as part of a family of zinc-dependent proteases (metzincin family) that play important roles in a variety of normal and pathological conditions, including arthritis and cancer.[15,16] Although expression of *ADAMTS16* in some organs has been reported, the relationship with cancers, including ESCC, has not been studied.

Materials and Methods

Tissue samples. For SAGE analysis, one primary ESCC (75-year-old male, T2N0M0) sample was used (Fig. 1). We confirmed microscopically that the tumor specimens consisted mainly (>80%) of carcinoma tissue. For quantitative RT-PCR analysis, 20 ESCC tissue samples and corresponding non-neoplastic mucosa samples were used. For Western blot analysis, four ESCC tissue samples and corresponding non-neoplastic mucosa samples were used. The samples were obtained from surgeries at Hiroshima University Hospital and affiliated hospitals. Samples were frozen immediately in liquid nitrogen and stored at −80°C until use. Fifteen kinds of normal tissue samples, including heart, lung, esophagus, stomach, small intestine, colon, liver, pancreas, kidney, bone marrow, peripheral

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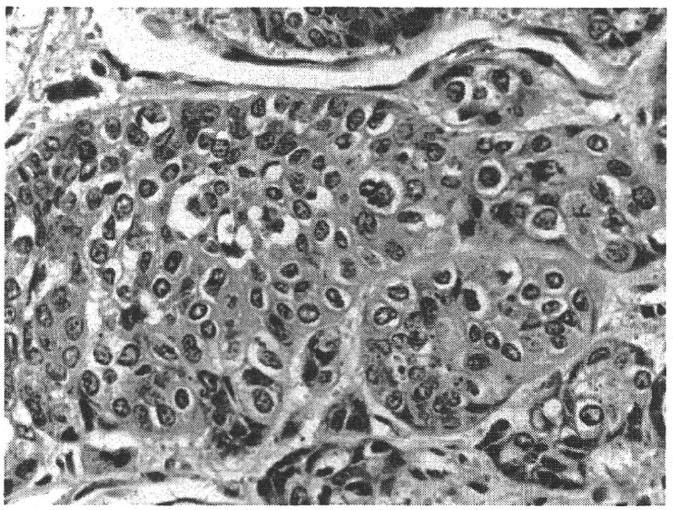

Fig. 1. Histological features of the esophageal squamous cell carcinoma sample analyzed by Serial Analysis of Gene Expression. The formalin-fixed, paraffin-embedded section was stained with H&E.

leukocytes, spleen, skeletal muscle, brain, and spinal cord, were purchased from Clontech (Palo Alto, CA, USA). Histological classification was based on the World Health Organization system. Tumor staging was done according to the TNM stage grouping system.[17] 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.

Serial analysis of gene expression. SAGE was carried out according to SAGE protocol version 1.0e (June 23, 2000). Tags were extracted from the raw sequence data with SAGE2000 analysis software version 4.12, kindly provided by Dr. Kenneth W. Kinzler (Ludwig Center for Cancer Genetics and Therapeutics and Howard Hughes Medical Institute, Johns Hopkins Kimmel Cancer Center, Baltimore, MD, USA).

Quantitative RT-PCR. Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and 1 μg total RNA was converted to cDNA with a First Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ, USA). PCR was carried out with a SYBR Green PCR Core Reagents Kit (Applied Biosystems, Foster City, CA, USA). *ADAMTS16* primer sequences were 5′-TCT CAT AGG AGT CGC CTC TGC-3′ and 5′-CGA GTG GAG CCC TCA CAG AA-3′. Squamous cell carcinoma antigen A1 (*SCCA1*) primer sequences were 5′-GAA TGG TGG ATA TCT TCA ATG GG-3′ and 5′-GAT AGC ACG AGA CCG CGG-3′. Real-time detection of the emission intensity of SYBR Green bound to double-stranded DNA was done with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as described previously.[18] Actin-beta-specific PCR products were amplified from the same RNA samples and served as internal controls.

Cell line and RNAi. Human esophageal cancer-derived cell lines, TE1, TE3, TE5, TE7, and TE13, were kindly provided by Dr. Tetsuro Nishihara (Tohoku University School of Medicine, Miyagi, Japan).[19] All cell lines were maintained in RPMI-1640 (Nissui Pharmaceutical, Tokyo, Japan) containing 10% FBS (Whittaker, Walkersville, MD, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. To knockdown the endogenous ADAMTS16, RNAi was carried out. siRNA oligonucleotides for ADAMTS16 and a negative control were purchased from Invitrogen (Carlsbad, CA, USA). Three independent oligonucleotides were used for ADAMTS16 siRNA. The ADAMTS16 siRNA1 sequence was 5′-CCA GUA UUA UCA CAU GGU CAC CAU U-3′. The ADAMTS16 siRNA2 sequence was 5′-ACA GAG ACC UGA AGU UUC AAG UAA A-3′. The ADAMTS16 siRNA3 sequence was 5′-GAG UAU AAG UCU UGC UUA CGG CAU A-3′. Transfection was carried out using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. Briefly, 60 pmol siRNA and 10 μL Lipofectamine RNAiMAX were mixed in 1 mL RPMI medium (10 nmol/L final siRNA concentration). After 20 min of incubation, the mixture was added to the cells and these were plated on dishes for each assay. Forty-eight hours after transfection, cells were analyzed for all experiments.

Western blot analysis. For Western blot analysis, tissue samples or cells were lysed as described previously.[20] 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, then subjected to 8% SDS-PAGE followed by electrotransfer onto a nitrocellulose filter. The filter was incubated with the primary antibody against ADAMTS16 (rabbit polyclonal, dilution 1:500; Abcam, Cambridge, UK). Peroxidase-conjugated antirabbit IgG was used in the secondary reaction. Immunocomplexes were visualized with an ECL Western Blot Detection System (Amersham Biosciences). β-actin antibody (Sigma Chemical, St. Louis, MO, USA) was also used as a loading control.

Cell growth and *in vitro* invasion assays. The cells were seeded at a density of 2000 cells per well in 96-well plates. Cell growth was monitored after 1 and 2 days by MTT assay.[21] Modified Boyden chamber assays were carried out to examine invasiveness. Cells were plated at 10 000 cells per well in RPMI-1640 medium plus 1% serum in the upper chamber of a Transwell insert (8 μm pore diameter; Chemicon, Temecula, CA, USA) coated with Matrigel. Medium containing 10% serum was added in the bottom chamber. After 1 and 2 days, cells in the upper chamber were removed by scraping, and the cells remaining on the lower surface of the insert were stained with CyQuant GR dye (Chemicon, Temecula, CA, USA) to assess the number of cells.

Statistical methods. Correlations between clinicopathologic parameters and *ADAMTS16* mRNA expression were analyzed by Fisher's exact test. A P value of <0.05 was considered statistically significant.

Results

Generation of SAGE data and comparison of expression patterns in ESCC and normal squamous esophagus. A total of 14 430 tags was generated, including 5765 that were unique. Then we compared SAGE tags from the ESCC sample with those from normal squamous esophagus (Gene Expression Omnibus accession number, GSM52501), which contained a total of 50 508 tags including 14 835 unique tags. The 20 most upregulated tags and the 20 most downregulated tags are shown in Tables 1 and 2. The upregulated tags included *ADAMTS16*, immunoglobulin heavy constant gamma 1 (*IGHG1*), 2-oxoglutarate and iron-dependent oxygenase domain containing 1 (*OGFOD1*), nuclear transport factor 2 (*NUTF2*), and RING1 and YY1 binding protein (*RYBP*), whose expressions have not been investigated in ESCC. The downregulated tags included S100 calcium binding protein A9 (*S100A9*), keratin 4 (*KRT4*), cystatin B (*CSTB*), exportin 7 (*XPO7*), keratin 6C (*KRT6C*), and epithelial membrane protein 1 (*EMP1*). Downregulation of some of these genes has been reported previously.[13] To identify novel biomarkers for ESCC diagnosis and novel targets for ESCC treatment, we focused on genes that were upregulated in the ESCC sample. Of the upregulated genes, we decided to analyze *ADAMTS16* expression because the amino acid sequence of the ADAMTS16 protein suggests that it might be secreted.

mRNA expression of ADAMTS16. Because genes expressed at high levels in tumors and at greatly reduced levels in normal tissues are ideal diagnostic markers and therapeutic targets,[4]

Table 1. Twenty most upregulated tags in esophageal squamous cell carcinoma (ESCC) compared to normal squamous esophagus (normal)

Tag sequence	Tags per million		Symbol	Description
	ESCC	Normal		
TCCCCTACAT	2564† (37)‡	0 (0)	*ADAMTS16*	ADAM metallopeptidase with thrombospondin type 1 motif, 16
GAAATAAAGC	2495 (36)	0 (0)	*IGHG1*	Immunoglobulin heavy constant gamma 1 (G1m marker)
TTCGGTTGGT	2148 (31)	0 (0)	*OGFOD1*	2-Oxoglutarate and iron-dependent oxygenase domain containing 1
AGGCATTGAA	5336 (77)	20 (1)	*NUTF2*	Nuclear transport factor 2
CAGTTACAAA	5544 (80)	40 (2)	*RYBP*	RING1 and YY1 binding protein
TGGAAATGAC	1317 (19)	0 (0)	*COL1A1*	Collagen, type I, alpha 1
GGCGTTTAGA	2079 (30)	20 (1)	No match	No match
ACCAAAAACC	1663 (24)	20 (1)	*COL1A1*	Collagen, type I, α1
GGCAGCACAA	1455 (21)	20 (1)	*NBEAL2*	Neurobeachin-like 2
TTTATTAGAA	1455 (21)	20 (1)	*CCDC75*	Coiled-coil domain containing 75
AGCCAAAAAA	2980 (43)	40 (2)	*MAP3K12*	Mitogen-activated protein kinase kinase kinase 12
GCTTTCATTG	2495 (36)	40 (2)	*NUCKS*	Nuclear casein kinase and cyclin-dependent kinase substrate 1
			GPX2	Glutathione peroxidase 2 (gastrointestinal)
ATGTGAAGAG	901 (13)	0 (0)	*SPARC*	Secreted protein, acidic, cysteine-rich (osteonectin)
CTCCCCCAAA	693 (10)	0 (0)	*KLK10*	Kallikrein-related peptidase 10
			IGHA2	Immunoglobulin heavy constant α2 (A2m marker)
GCTTAAAAAA	693 (10)	0 (0)	*CORO1C*	Coronin, actin binding protein, 1C
ATTTGAGAGT	624 (9)	0 (0)	*MYH9*	Myosin, heavy chain 9, non-muscle
CTTTATTCCA	624 (9)	0 (0)	*WWC2*	WW and C2 domain containing 2
TCAAGCCATC	624 (9)	0 (0)	*BLMH*	Bleomycin hydrolase
			PCYT2	Phosphate cytidylyltransferase 2, ethanolamine
TTTTCCAATT	624 (9)	0 (0)	*UTP3*	UTP3, small subunit (SSU) processome component, homolog (*S. cerevisiae*)
TTGCTCACAA	1178 (17)	20 (1)	*ABHD12B*	Abhydrolase domain containing 12B

†Absolute tag counts are normalized to 1 000 000 total tags/sample. ‡Number in parentheses indicates the absolute tag counts.

Table 2. Twenty most downregulated tags in esophageal squamous cell carcinoma (ESCC) compared to normal squamous esophagus (normal)

Tag sequence	Tags per million		Symbol	Description
	ESCC	Normal		
GTGGCCACGG	0 (0)	25 283† (1277)‡	*S100A9*	S100 calcium binding protein A9 (calgranulin B)
GGCAGAGAAG	0 (0)	8454 (427)	*KRT4*	Keratin 4
ATGAGCTGAC	0 (0)	3762 (190)	*CSTB*	Cystatin B (stefin B)
			XPO7	Exportin 7
GAAGCACAAG	0 (0)	2475 (125)	*KRT6C*	Keratin 6C
TAATTTGCAT	0 (0)	2455 (124)	*EMP1*	Epithelial membrane protein 1
			GNA13	Guanine nucleotide binding protein (G protein), α 13
AAAGCGGGGC	0 (0)	2356 (119)	*KRT13*	Keratin 13
TGTGTTGAGA	0 (0)	2257 (114)	*EEF1A1*	Eukaryotic translation elongation factor 1 α 1
CACAAACGGT	0 (0)	2079 (105)	*TSPAN9*	Tetraspanin 9
			RPS27	Ribosomal protein S27
TGGTGTTGAG	0 (0)	1841 (93)	*RPS18*	Ribosomal protein S18
GCCAATCCAG	0 (0)	1802 (91)	*CRNN*	Cornulin
GGCAAGCCCC	0 (0)	1782 (90)	*RPL10A*	Ribosomal protein L10a
			PTPRG	Protein tyrosine phosphatase, receptor type, G
AAGGAGATGG	0 (0)	1722 (87)	*RPL31*	Ribosomal protein L31
			ZNF434	Zinc finger protein 434
CTGTCACCCT	0 (0)	1564 (79)	*SPRR1A*	Small proline-rich protein 1A
			BTC	Betacellulin
TAAGGAGCTG	0 (0)	1485 (75)	*RPS26*	Ribosomal protein S26
			ANK2	Ankyrin 2, neuronal
ACCTGGAGGG	0 (0)	1386 (70)	*SBSN*	Suprabasin
			PCBP1	Poly(rC) binding protein 1
ACGTGTGTAA	0 (0)	1386 (70)	No match	No match
CAAATCCAAA	0 (0)	1366 (69)	No match	No match
GCCGAGGAAG	0 (0)	1346 (68)	*RPS12*	Ribosomal protein S12
			NCKAP5L	NCK-associated protein 5-like
TGTGCTAAAT	0 (0)	1346 (68)	*USP36*	Ubiquitin specific peptidase 36
			RPL34	Ribosomal protein L34
GGGTCTGAGG	0 (0)	1307 (66)	*SLURP1*	Secreted LY6/PLAUR domain containing 1
			PTPRG	Protein tyrosine phosphatase, receptor type, G

†The absolute tag counts are normalized to 1 000 000 total tags/sample. ‡Number in parentheses indicates the absolute tag counts.

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quantitative RT-PCR of *ADAMTS16* was carried out in 20 ESCC samples and in 15 kinds of normal tissue (liver, kidney, heart, colon, brain, bone marrow, skeletal muscle, lung, small intestine, spleen, spinal cord, stomach, pancreas, leukocyte, and esophagus) (Fig. 2a). Among the various normal tissues, obvious *ADAMTS16* expression was found in normal brain, spinal cord, pancreas, and kidney, as reported elsewhere.[22] Expression of *ADAMTS16* in these normal tissues was highest in spinal cord; however, in ESCC, high levels of *ADAMTS16* mRNA expression (more than twice the mRNA expression of spinal cord) were found in eight out of 20 cases (40%). *ADAMTS16* expression in two ESCC cases (Cases 16 and 17) was 10-fold higher than in spinal cord. High levels of *ADAMTS16* mRNA expression were not correlated with any clinicopathologic characters (data not shown). Among five cases at stage I ESCC, a high level of *ADAMTS16* mRNA was detected in one case (20%). These results indicate that *ADAMTS16* expression is highly specific for cancer, at least in ESCC.

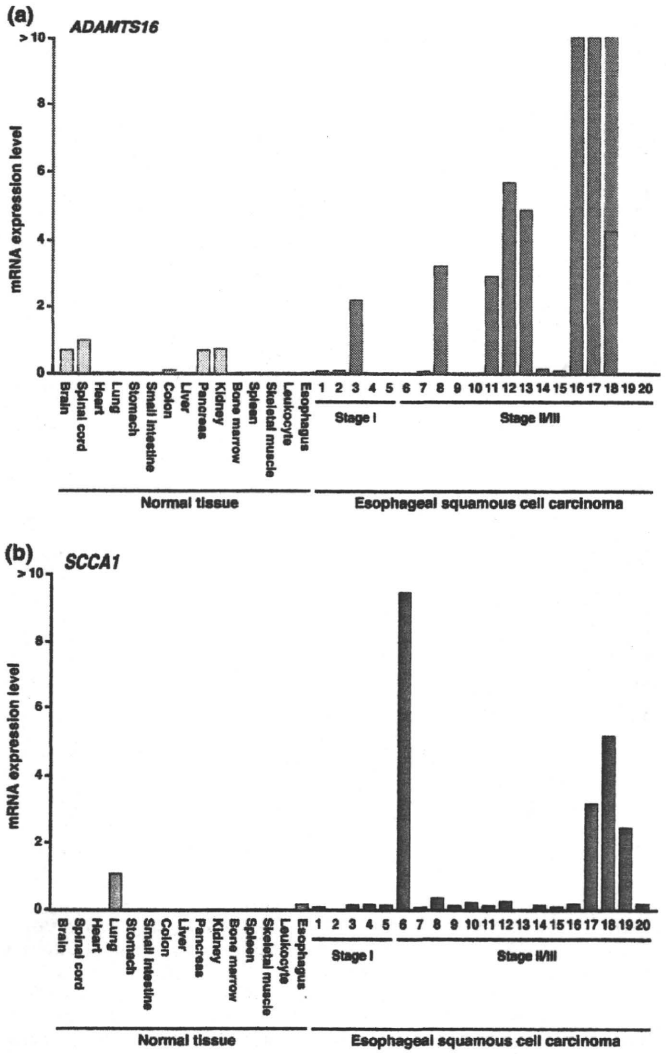

Fig. 2. Quantitative RT-PCR analysis of *ADAMTS16* and *SCCA1* in 15 kinds of normal tissues and 20 esophageal squamous cell carcinoma tissues. (a) mRNA expression level of *ADAMTS16*. The units are arbitrary, and we calculated *ADAMTS16* mRNA expression by standardization of the expression in normal spinal cord to 1.0. (b) mRNA expression level of *SCCA1*. The units are arbitrary, and we calculated *SCCA1* mRNA expression by standardization of the expression in normal lung to 1.0.

Serum squamous cell carcinoma antigen (SCC antigen) detected in the normal squamous epithelium and in ESCC has been considered a useful tumor marker for ESCC.[23] SCC antigen predicts recurrence or progression of the disease and has been used extensively for this purpose. However, clinical use of this marker has been restricted because of lack of sensitivity.[24] Therefore, there is an urgent need for new biomarkers for ESCC. To evaluate the usefulness of determining ADAMTS16 expression as a tumor marker, we measured expression levels of SCC antigen and compared them with ADAMTS16 levels. Because a measurement system for serum levels of ADAMTS16 is not available, we investigated the mRNA expression levels of *SCCA1*, which encodes SCC antigen, by quantitative RT-PCR (Fig. 2b). In 15 kinds of normal tissue, expression of *SCCA1* was highest in lung; however, in ESCC, high levels of *SCCA1* mRNA expression (more than twice the mRNA expression levels of lung) were found in four of 20 cases (20%). Among five cases at stage I ESCC, high levels of *SCCA1* mRNA were not detected. These results indicate that ADAMTS16 might serve as a more sensitive biomarker than SCC antigen. We calculated the ratio of *ADAMTS16* mRNA expression levels between ESCC tissue (T) and corresponding non-neoplastic mucosa (N). T/N ratios >2-fold higher were considered to represent overexpression. *ADAMTS16* overexpression was observed in 13 of 20 ESCC cases (65%). Among five cases at stage I ESCC, *ADAMTS16* overexpression was found in one case (20%). We then investigated the relation of *ADAMTS16* expression to clinicopathologic characters (Table 3). We found that *ADAMTS16* overexpression correlated to the advanced T classification and tumor stage.

ADAMTS16 protein expression. Analysis of the amino acid sequence of the ADAMTS16 protein suggests that it might be secreted. To investigate whether ADAMTS16 is a secreted protein, we used Western blot analysis in five esophageal cancer cell lines. Moderate to high ADAMTS16 expression was noted in TE1, TE3, and TE5 cells as a band of approximately 136 kDa, and the other two remaining cell lines (TE7 and TE13) had low or absent ADAMTS16 expression (Fig. 3a). Next, we examined the transition of ADAMTS16 expression by Western blot analysis of cell extracts of TE5 transfected with *ADAMTS16* specific siRNAs. Three types of siRNAs (siRNA1–3) were transfected into TE5. The expression of ADAMTS16 in TE5 was substantially suppressed by treatment with siRNA2

Table 3. Relationship between ADAMTS16 expression and clinicopathologic characteristics in esophageal squamous cell carcinoma

	ADAMTS16 expression		*P* value*
	Overexpression	No overexpression	
Age (years)			
≤65	8 (80%)	2 (20%)	0.3498
>65	5 (50%)	5 (50%)	
Sex			
Male	11 (69%)	5 (31%)	0.5868
Female	2 (50%)	2 (50%)	
T classification			
T1	2 (29%)	5 (71%)	0.0215
T2/3	11 (85%)	2 (15%)	
N classification			
N0	3 (43%)	4 (57%)	0.1736
N1	10 (77%)	3 (23%)	
Stage			
Stage I	1 (20%)	4 (80%)	0.0307
Stage II/III	12 (80%)	3 (20%)	

*Fisher's exact test. N, node; T, tumor.

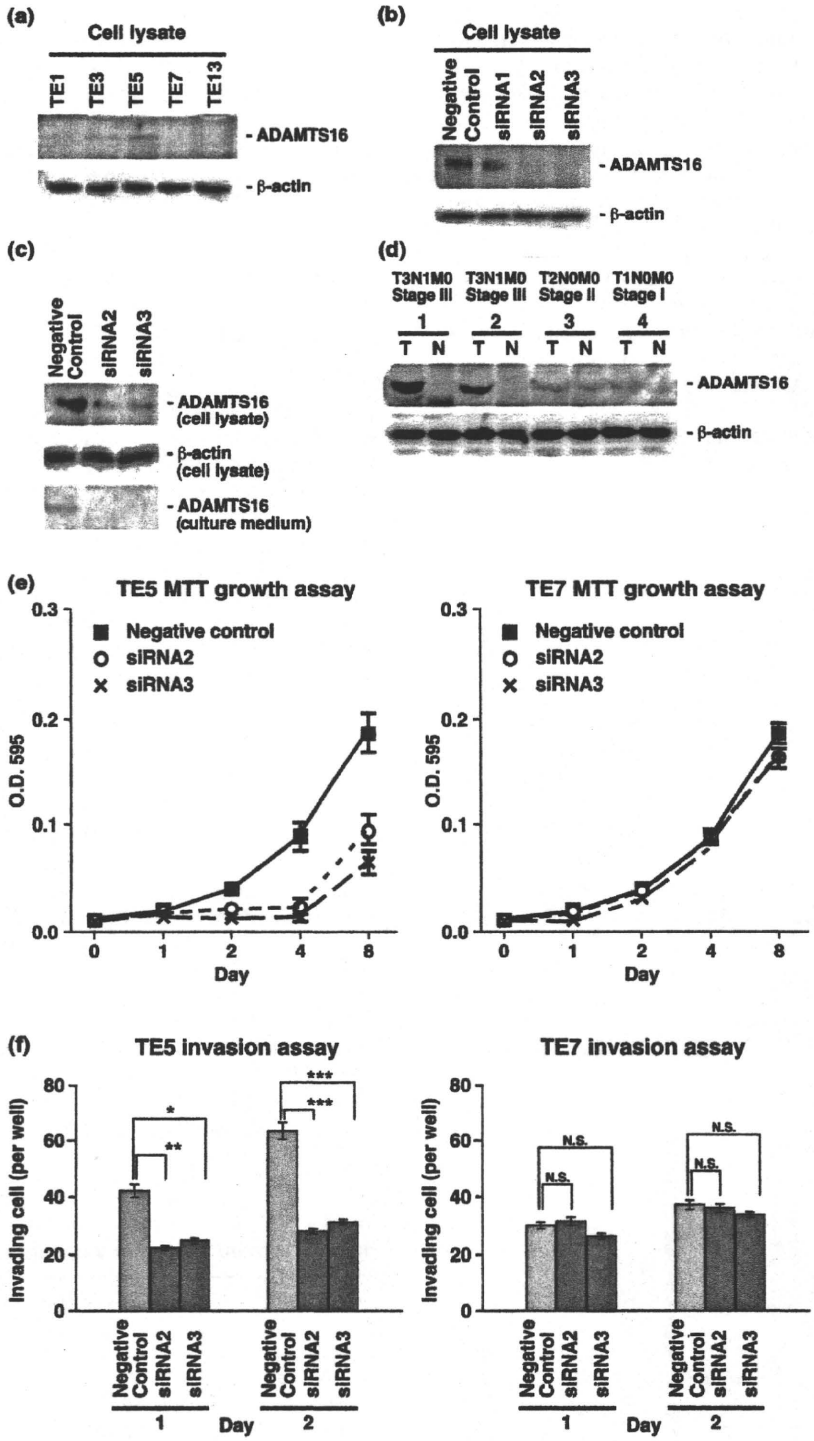

Fig. 3. ADAMTS16 protein expression and functional analysis. (a) Western blot analysis of ADAMTS16 in five esophageal squamous cell carcinoma (ESCC) cell lines. (b) Western blot analysis of ADAMTS16 in cell lysates from TE5 cells transfected with the negative control siRNA and ADAMTS16 siRNA (siRNA1–3). (c) Western blot analysis of ADAMTS16 in cell lysates and culture media from TE5 cells transfected with the negative control siRNA and ADAMTS16 siRNA (siRNA2 and 3). (d) Western blot analysis of ADAMTS16 in four ESCC samples (T) and corresponding non-neoplastic mucosa samples (N). (e) Effect of ADAMTS16 knockdown on cell growth of TE5 and TE7 cells. Cell growth was assessed by an MTT assay at 1, 2, 4, and 8 days after seeding on 96-well plates. Bars and error bars, mean and SE of three different experiments. O.D., optical density. (f) Effect of ADAMTS16 knockdown on cell invasion of TE5 and TE7 cells. TE5 and TE7 cells transfected with negative control siRNA and ADAMTS16 siRNA (siRNA2 and 3) were incubated in Boyden chambers. After 1 and 2 days, invading cells were counted. Bars and error bars, mean and SE of three different experiments. N.S., not significant. $*P = 0.0006$; $**P = 0.0003$; $***P < 0.0001$.

and siRNA3, but not with siRNA1 (Fig. 3b). Therefore, to knock down the endogenous ADAMTS16, we used siRNA2 and siRNA3 in the following experiments. A Western blot was carried out of siRNA (siRNA2 and siRNA3)-transfected TE5 cell extracts and culture media (Fig. 3c). In negative control siRNA-transfected TE5 cells, ADAMTS16 protein was detected in

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culture media as well as cell extracts; however, in ADAMTS16 siRNA-transfected TE5 cells, ADAMTS16 protein was low or absent in culture media as well as cell extracts. These results clearly indicate that ADAMTS16 is a secreted protein.

Next, expression of ADAMTS16 protein was analyzed by a Western blot of four ESCC tissue samples and corresponding non-neoplastic mucosa samples (Fig. 3d). Among the four ESCC samples, ADAMTS16 protein expression was detected in all; however, of the four corresponding non-neoplastic mucosa samples, ADAMTS16 protein expression was found in only one sample. These results indicate that ADAMTS16 protein is overexpressed in ESCC tissue, and can serve as a serum tumor marker for ESCC.

Effect of ADAMTS16 inhibition on cell growth and invasive activity of esophageal cancer cells. High levels of *ADAMTS16* mRNA expression were correlated with T classification of ESCC tissues; however, the biological significance of ADAMTS16 in ESCC has not been studied. To investigate the possible antiproliferative effects of ADAMTS16 knockdown, we carried out an MTT assay 8 days after siRNA transfection (Fig. 3e). TE5 cells were selected for high ADAMTS16 expression. ADAMTS16 siRNA2-transfected and siRNA3-transfected TE5 cells showed significantly reduced viability relative to negative control siRNA-transfected TE5 cells. We carried out the same assay using one additional esophageal cancer cell line that did not express ADAMTS16 (TE7). Reduced cell viability was not observed in siRNA2- or siRNA3-transfected TE7 cells compared with negative control siRNA-transfected TE7 cells.

Next, to determine the possible role of ADAMTS16 in the invasiveness of esophageal cancer cells, we used a Transwell invasion assay (Fig. 3f). On day 1, although there was no difference in cell viability between ADAMTS16 knockdown TE5 cells and negative control siRNA-transfected TE5 cells, the invasiveness of ADAMTS16 knockdown TE5 cells was 40% less than that of the negative control siRNA-transfected TE5 cells. On day 2, the invasiveness of ADAMTS16 knockdown TE5 cells was 50% less than that of the negative control siRNA-transfected TE5 cells; however, as ADAMTS16 knockdown cells showed significantly reduced cell viability, the cell number difference observed in the invasion assay might be caused by the reduced cell viability. In contrast, invasion ability was not significantly different between ADAMTS16 knockdown TE7 cells and negative control siRNA-transfected TE7 cells. These results indicate that ADAMTS16 stimulates cell growth and invasion in esophageal cancer cells.

Discussion

In spite of improvement to modern surgical techniques and adjuvant CRT, ESCC is known to reveal the worst prognosis among malignant tumors. Therefore, it is now urgently required to develop novel diagnostic biomarkers and therapeutic targets for a better choice of adjuvant treatment modalities for individual patients. In the present study, we carried out a genome-wide expression profile analysis of one ESCC tissue sample by SAGE, and identified upregulated and downregulated genes in ESCC. Among these, we further investigated *ADAMTS16*. Quantitative RT-PCR revealed that *ADAMTS16* mRNA expression was frequently upregulated in ESCC, and was narrowly restricted in normal tissues. Western blot analysis also showed upregulation of ADAMTS16 protein in ESCC. Furthermore, ADAMTS16 protein was detected in culture media from TE5 cells. Taken together, these results suggest that ADAMTS16 has potential as a serum tumor marker for ESCC. Because the frequency of high levels of *ADAMTS16* mRNA expression (40%) was greater than the frequency of high levels of *SCCA1* mRNA expression (20%), serum concentrations of ADAMTS16 might serve as a sensitive biomarker for ESCC. In contrast, because *ADAMTS16* mRNA overexpression was correlated with advanced T classification and tumor stage, serum concentrations of ADAMTS16 might not be suitable for early detection of ESCC. Serum concentrations of ADAMTS16 should be measured in patients with ESCC.

In the present study, *ADAMTS16* mRNA overexpression correlated to the advanced T classification and tumor stage. Knockdown of ADAMTS16 by RNAi inhibited the cell growth and invasion ability of TE5 cells. Because expression of ADAMTS16 was highly specific to ESCC, it could be a good therapeutic target with less adverse effects for ESCC. Although the function of ADAMTS16 is poorly understood, members of the metzincin family are known to process a number of growth factors, cytokines and signaling molecules in addition to matrix substrates.[25] However, it has been reported that the forced expression of ADAMTS16 has no effect on expression levels of most of the ADAMTS, TIMP, and MMP genes. In the present study, we also used ELISA to measure levels of epidermal growth factor (EGF) and transforming growth factor (TGF)-α in culture media from TE5 cells transfected with ADAMTS16 siRNA and negative control siRNA; however, levels of EGF and TGF-α were not significantly different (data not shown). Therefore, growth factors or cytokines, such as EGF or TGF-α, are not likely to be involved in mechanisms of cell growth inhibition and invasion ability following knockdown of ADAMTS16.

Although ADAMTS16 protein upregulation was observed in ESCC tissues by Western blot analysis, expression and distribution of ADAMTS16 protein in ESCC tissues remains unclear. Therefore, immunohistochemical analysis should be undertaken. Unfortunately, the antibody against ADAMTS16 used in the present study is not suitable for immunostaining because the antibody against ADAMTS16 detected multiple bands on Western blots. Production of a specific antibody against ADAMTS16 is required. Furthermore, ADAMTS16 expression at mRNA and protein levels should be examined in several more tissues from stage I ESCC in the near future.

In addition to *ADAMTS16*, other upregulated and downregulated genes in ESCC were found. The upregulated group of genes identified by SAGE contains genes whose expression has not been investigated in ESCC. Upregulation of two genes related to the immunoglobulin heavy chain (*IGHG1* and *IGHA2*) was found in the present study. Previously, genes involved in the immune response have been shown as characteristically upregulated in long-term ESCC survivors who were treated with CRT.[26] Therefore, the ESCC case analyzed by SAGE in the present study might be sensitive to CRT. *OGFOD1* is a 2-oxoglutarate and Fe(II)-dependent oxygenase, a class of enzymes that catalyze a variety of reactions typically involving the oxidation of an organic substrate using a dioxygen molecule.[27] To our knowledge, association between cancer and *OGFOD1* has not been investigated. *NUTF2* encodes nuclear transport factor 2 (NTF2), which is a small GDP Ran binding protein. The main function of NTF2 is to facilitate transport of certain proteins into the nucleus through interaction with nucleoporin FxFG.[28] It is also involved in regulating multiple processes, including cell cycle and apoptosis.[29] However, no studies have analyzed NTF2 expression in human cancer, including ESCC. *RYBP* is a member of the polycomb group, and it has been reported that RYBP interacts with MDM2 and decreases MDM2-mediated p53 ubiquitination, leading to stabilization of p53 and an increase in p53 activity.[30] RYBP induces cell cycle arrest and is involved in the p53 response to DNA damage. Expression of RYBP is decreased in hepatocellular carcinoma and lung cancer tissues.[30] Therefore, upregulation of RYBP should be confirmed in a large number of ESCC cases. In contrast, downregulated genes identified by SAGE in the present study were

similar to genes previously reported as downregulated in ESCC.[13]

In conclusion, our present SAGE data provide a list of genes potentially associated with ESCC. Because our list is based on one ESCC case, expression analysis in a large number of cases is required. A high level of *ADAMTS16* expression was detected in ESCC, and expression of *ADAMTS16* was narrowly restricted. Production of a specific antibody against ADAMTS16 protein and establishment of a measurement system for serum samples are needed to clarify whether ADAMTS16 serves as a serum marker for early detection and a good therapeutic target for ESCC.

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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.[1] 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

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

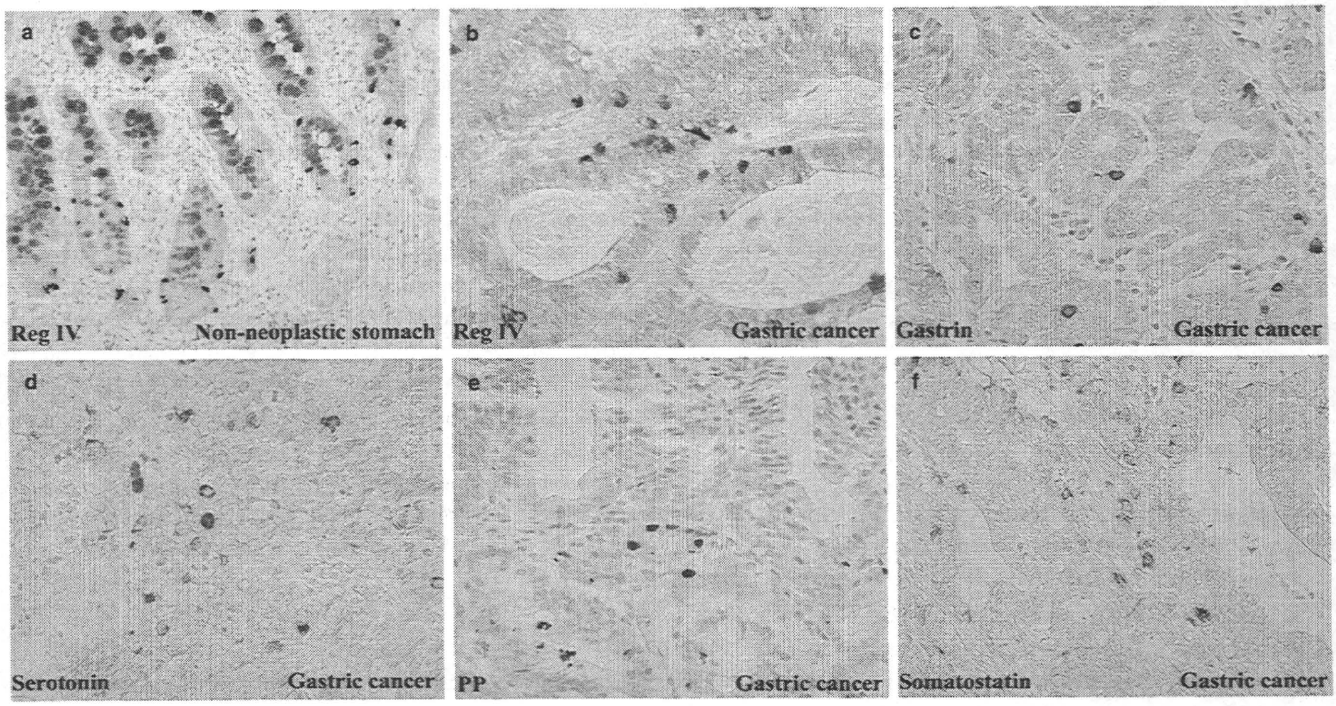

Figure 1 Immunohistochemical staining of Reg IV and neuroendocrine (NE) hormones. Reg IV showed positive staining in both goblet cell vesicles in goblet cells and the perinuclear region of neuroendocrine cells at the base of intestinal metaplasia (**a**). Reg IV showed positive staining in the perinuclear 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**).

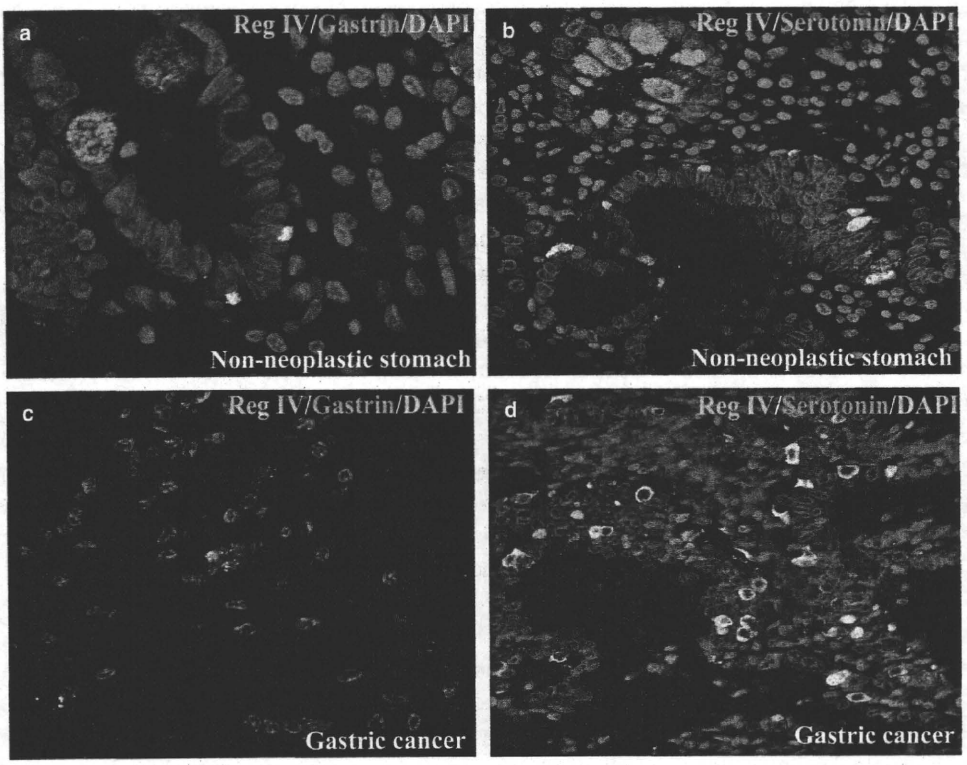

Figure 2 Double immunofluorescence staining revealed some non-neoplastic stomach cells with co-expression between Reg IV and gastrin (**a**), and Reg IV and serotonin (**b**). Some gastric cancer cells showed co-expression between Reg IV and gastrin (**c**), and Reg IV and serotonin (**d**). Cells were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

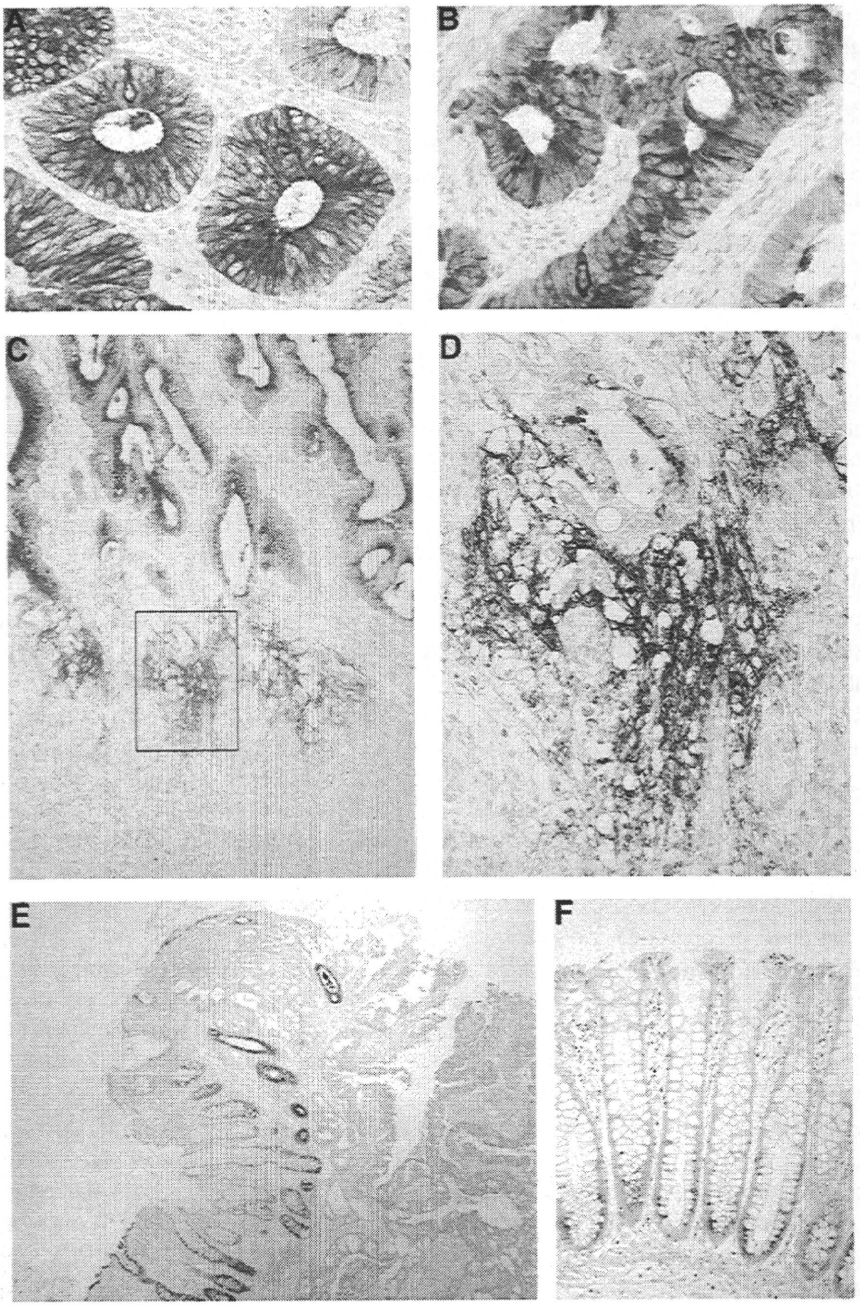

Figure 1. Immunohistochemical analysis of olfactomedin 4 in CRC and peritumoral mucosa. (A) Immunostaining of olfactomedin 4 in well-differentiated CRC. Staining of olfactomedin 4 was detected in the cytoplasm of tumor cells. Original magnification x400. (B) Immunostaining of olfactomedin 4 in moderately 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

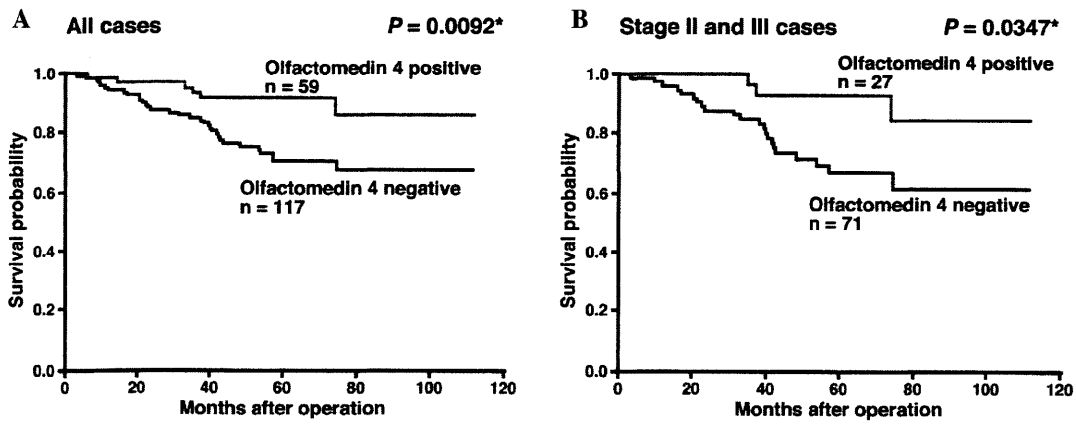

Figure 2. Survival of patients with CRC. (A) Kaplan-Meier curves of the CRC patients (all stages) with olfactomedin 4-negative or olfactomedin 4-positive tumors. (B) Kaplan-Meier curves of stage II and stage III CRC patients with olfactomedin 4-negative and olfactomedin 4-positive tumors. *Log-rank test.

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.

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