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RUNX3 expression correlates with chief cell differentiation in human gastric cancers

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Summary. RUNX3 is a novel tumor suppressor in gastric carcinogenesis and an important factor for differentiation of chief cells in the normal gastric fundic mucosa. In this study, we confirmed RUNX3 immunolocalization in the fundic gland (bottom part) but minimum in surface mucous cell epithelium (top part) in the isolated gland from fundic mucosa. We also analyzed RUNX3 expression by immunohistochemistry in 102 gastric cancers and made a histological assessment of the expression of differentiation markers to evaluate interrelations. Among them, 45 and 57 cases were judged to be RUNX3 positive and negative, respectively, and 33 and 69 cases were pepsinogen I positive and negative, with no link to histological types. RUNX3 expression was significantly associated with that of pepsinogen I ($P<0.001$), but not mucins, including MUC5AC and MUC6, or the parietal or intestinal phenotypes. In conclusion, the present study showed, for the first time to our knowledge, a relation between RUNX3 and pepsinogen I expression in human gastric cancers. RUNX3 is strongly associated with chief cell phenotypic expression in human gastric cancers, as well as in normal gastric mucosa, and could be considered to play an important role in maintaining the chief cell phenotype.

Key words: Gastric cancer, RUNX3, Pepsinogen I, Chief cell differentiation

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Introduction

A lack of RUNX3 (runt-related family of transcription factor 3) functions is causally related to the genesis and progression of human gastric cancer, indicating roles as a novel tumor suppressor (Li et al., 2002). RUNX3 shows remarkable down regulation in gastric cancers compared to surrounding mucosa, correlating with cancer stage progression. Furthermore, RUNX3 expression is reduced in intestinal metaplasia, commonly considered as a precancerous state, as compared with normal mucosa (Li et al., 2002). In addition, the gastric mucosa of Runx3-null mice is hyperplastic with suppression of transforming growth factor ß (TGF-ß)-mediated apoptosis. Analyses with a primary culture system for gastric epithelial cells also demonstrated Runx3(-/-) gastric epithelial cells to have low sensitivity to the growth-inhibiting and apoptosis-inducing activities of TGF-ß, so that RUNX3 is generally considered a major growth regulator of gastric epithelial cells (Li et al., 2002; Fukamachi et al., 2004).

Histochemical and immunohistochemical analyses have been developed for identification of cellular mucin in both gastric and intestinal epithelium. Recently, mucin-type molecules have been revealed to consist of a core protein moiety (apomucin), where a number of carbohydrate chains are attached to serines and threonines by glycosidic bonds. In the gastrointestinal tract, the MUC5AC and MUC6 genes are mainly expressed in gastric foveolar mucosa and pyloric glands, respectively. Pepsinogen I and the proton pump are expressed in the chief cells (Huang et al., 1988; Takahashi, 1992) and the parietal cells (Takubo et al.,

2002) of gastric fundic glands, respectively. The MUC2 gene encodes a typical secretory gel-forming mucin, which represents the predominant form in human intestinal and colon tissues (Seregni et al., 1997). Cells of intestinal absorptive cell type demonstrate sucrase and intestinal-type alkaline phosphatase activity (Tatematsu et al., 1990). Several reports have indicated that it is possible to analyze the phenotypic expression of each gastric cancer cell using gastric and intestinal epithelial cell markers (Tatematsu et al., 1986, 1990, 1992; Koseki et al., 2000; Tajima et al., 2001; Kawachi et al., 2003) and various authors have demonstrated correlations between prognosis and such phenotypic markers in gastric cancers (Tsuchiya et al., 1995; Utsunomiya et al., 1998; Lee et al., 2001; Tajima et al., 2001; Baldus et al., 2002). However, there has been no report regarding the relationship between RUNX3 and mucin expression in gastric cancers. Also, information on the characteristics of gastric fundic gland cell types in gastric cancers is limited (Muller-Hocker and Rellecke, 2003). In the present study, we therefore analyzed RUNX3 expression in 102 gastric cancers, together with histological evaluation and assessment of mucin expression by immunohistochemistry. The main purpose was to evaluate the relation between RUNX3 expression and gastric differentiation, especially of chief cells.

Materials and methods

Samples and tissue collection

We examined 102 primary gastric cancers surgically resected at Aichi Cancer Center Hospital between 1992 and 2003. The patients were 60 men with an average age of 62.8±9.80 (SD) years (range, 49-79 years) and 42 women aged 61.3±11.1 years (range, 23-84 years). All specimens were fixed in 10% buffered formalin and embedded in paraffin, and then stained with hematoxylin-eosin (H&E). Histopathological classification was made into differentiated (papillary and well and moderately differentiated tubular adenocarcinomas) and undifferentiated (poorly differentiated adenocarcinomas and signet-ring cell carcinomas) types according to the Japanese Classification of Gastric Carcinomas (Japanese Gastric Cancer Association, 1998).

Gland isolation

Normal portions of resected epithelium were injected with 30 mM ethylene diamine tetraacetic acid in Hanks' balanced salt solution (EDTA-HBSS) submucosally and incubated at 37°C for 15 min in EDTA-HBSS solution in a water bath. Then the mucosa was scraped off with a scalpel. Isolated glands were washed in PBS, fixed in 70% ethanol for a few hours, dehydrated with 95% ethanol, and stored at -20°C until use (Tsukamoto et al., 2001, 2004). Groups of isolated classified fundic glands were transferred to 0.5-ml microfuge tubes under an inverted microscope (Axiovert 200, Carl Zeiss, Jena, Germany), ethanol fixed, and subjected to immunohistochemstry for pepsinogen I and RUNX3, as well as HE staining, as documented previously (Tsukamoto et al., 2001, 2004).

Immunohistochemistry

Immunohistochemical staining was carried out with monoclonal antibodies against the following antigens: Cdx2 (CDX2-88; 1:50, BioGenex, CA, USA); MUC5AC (CLH2; 1:500, Novocastra Laboratories, Newcastle upon Tyne, UK); MUC6 (CLH5; 1:500, Novocastra Laboratories); pepsinogen I (mouse monoclonal; 1:30,000) (Huang et al., 1988); the proton pump (H+, K+ ATPase) α subunit (2B6; 1:500, Medical & Biological Laboratories, Japan); RUNX3 (6E9; 1:3,000) (Ito et al., 2005); MUC2 (Ccp58; 1:500, Novocastra Laboratories); and villin (12; 1:20,000, Transduction Laboratories, Lexington, KY, USA). With regard to gastric and intestinal phenotypic markers, we used normal gastric mucosa and normal ileum as positive and negative controls. The precise procedures for immunohistochemical techniques were as previously described (Tsukamoto et al., 2005, 2006). Briefly, 5 μm-thick consecutive sections were deparaffinized and hydrated through a graded series of ethanol. After inhibition of endogenous peroxidase activity by immersion in 3% H_2O_2/methanol solution, antigen retrieval was conducted for detection of binding of the above-mentioned antibodies with 10 mM citrate buffer (pH 6.0) in a microwave oven for 10 minutes at 98°C, or a pressure cooker for 10 minutes at 121°C. Sections were incubated with primary antibodies, thoroughly washed in phosphate-buffered saline (PBS), then incubated with biotinylated secondary antibody, followed by the avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Inc., Burlingame, CA, USA). Finally, immune complexes were visualized by incubation with 0.01% H_2O_2 and 0.05% 3,3'-diaminobenzidine tetrachloride (DAB). Nuclear counterstaining was accomplished with Mayer's hematoxylin. The results for each antibody staining were evaluated in terms of the percentage of positively stained cancer cells, with 10% and above considered positive, as previously described (Kawachi et al., 2003; Mizoshita et al., 2003).

Immunohistological markers for gastric cancer cells

MUC5AC and MUC6 are markers of gastric epithelial and pyloric gland cells respectively, whereas MUC2 and villin are typical of the intestinal epithelial cell phenotype (Tatematsu et al., 2003). Cdx2 is a homeobox gene related to intestinalization of gastric mucosa. Pepsinogen I and the proton pump α subunit are markers of chief cells and parietal cells, respectively, in

the gastric fundic glands.

Dual immunofluorescent staining

The procedure for double immunofluorescent staining was as previously documented (Suzuki et al., 2005). Briefly, sections were antigen-retrieved, incubated with anti-Pepsinogen I primary antibody (dilution 1:5000) overnight at 4°C, incubated with biotinylated secondary antibody, and then fluorescein isothiocyanate (FITC)-labled streptavidin (DAKO). To inactivate the antigenicity of the primary antibody, sections were heated in 10 mM citrate buffer (pH 6.0) in a microwave oven for 15 minutes at 90°C. After that sections were incubated with anti-RUNX3 primary antibody (dilution 1:500) for 1 hour at 37°C and with secondary antibody labeled with rhodamine (CHEMICON). Nuclei were counter-stained with 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes).

Statistical analysis

The data were analyzed by the Fischer's exact test. P-values <0.05 were considered as statistically significant.

RUNX3 and pepsinogen I mRNA expressions in gastric cancer cell lines

Three human gastric carcinoma cell lines (MKN45, MKN74, and THK1) were cultured in RPMI1640 supplemented with 10% fetal bovine serum (FBS). Total RNA was isolated and cDNA was synthesized using the Thermoscript RT-PCR System (Invitrogen, Carlsbad, CA, USA), which were subjected for 35 cycles of PCR analysis of RUNX3 and pepsinogen I mRNA expression using primers listed in Table 1 using acid ribosomal phosphoprotein PO (ARP) as an internal control.

Results

RUNX3 and pepsinogen I expression in nomal fundic glands

RUNX3 staining was detected mainly in the chief cells of fundic glands (Fig. 1). Staining was also detected in monocytes and lymphocytes in the stromal tissue. Pepsinogen I cytoplasmic staining was detected only in the chief cells of fundic glands (Fig. 1E), similar to RUNX3 cytoplasmic expression (Fig. 1D). Immunofluorescent dual staining revealed that pepsinogen I and RUNX3 merged mostly in the same cells in fundic gland (Fig. 2A-C). To further confirm the immunolocalization of pepsinogen I and RUNX3 proteins, isolated glands from fundic mucosa were used for their evaluation; these differentiation markers are limited to the basal region of the fundic gland but demonstrate mimimal expression in the upper part (Fig. 3).

Immunohistochemical analysis of RUNX3 in gastric cancers

Of the 102 gastric cancers, 45 and 57 cases were judged as RUNX3 positive and negative (Fig. 4), this feature appearing independently of the histological type (Table 2).

Correlation between RUNX3 and pepsinogen I expression in gastric cancers

Of the 102 gastric cancers, 33 and 69 cases were judged to be pepsinogen I positive and negative, respectively (Table 3). In the pepsinogen I-positive gastric cancers, the areas of pepsinogen I and RUNX3 positive staining were strongly correlated (Fig. 4), being consistent with immunofluorescent staining at least in part (Fig. 2D-F). The rates for RUNX3 positive cases in the pepsinogen I-positive and negative cancers were 79% and 26%, respectively, with a significant positive correlation (Table 3, $P<0.001$).

No relationship between RUNX3 expression and MUC5AC or MUC6

51 and 51 cases were judged to be MUC5AC positive and negative (Table 3). The rates for RUNX3 positive cases in the MUC5AC-positive and negative cancers were 41% and 47%, respectively, the difference not being significant. Also, 19 and 83 cases were judged to be MUC6 positive and negative (Table 3). The rates for RUNX3 positive cases in the MUC6-positive and

Table 1. Primer sequences for analysis of mRNA levels of Pepsinogen I and RUNX3 in human gastric cancer cell lines.

Genes	Directions	Sequences	Gene Bank accession numbers
Pepsinogen I	Upper	CAACCACAACCGCTTCAACCCTGAGGA	BC29055
	Lower	GCCGGTGCCGTAGGTGATGGAGACTGT	
RUNX3	Upper	GCCAACCCGTCCCCCTACCACCTCTACT	NM_004350
	Lower	GCTGCCGGCCACCATGGAGAACT	
ARP	Upper	CGAAGCCACGCTGCTGAACATGCTCAAC	M7885
	Lower	GCTGCCATTGTCGAACACCTGCTGGATG	

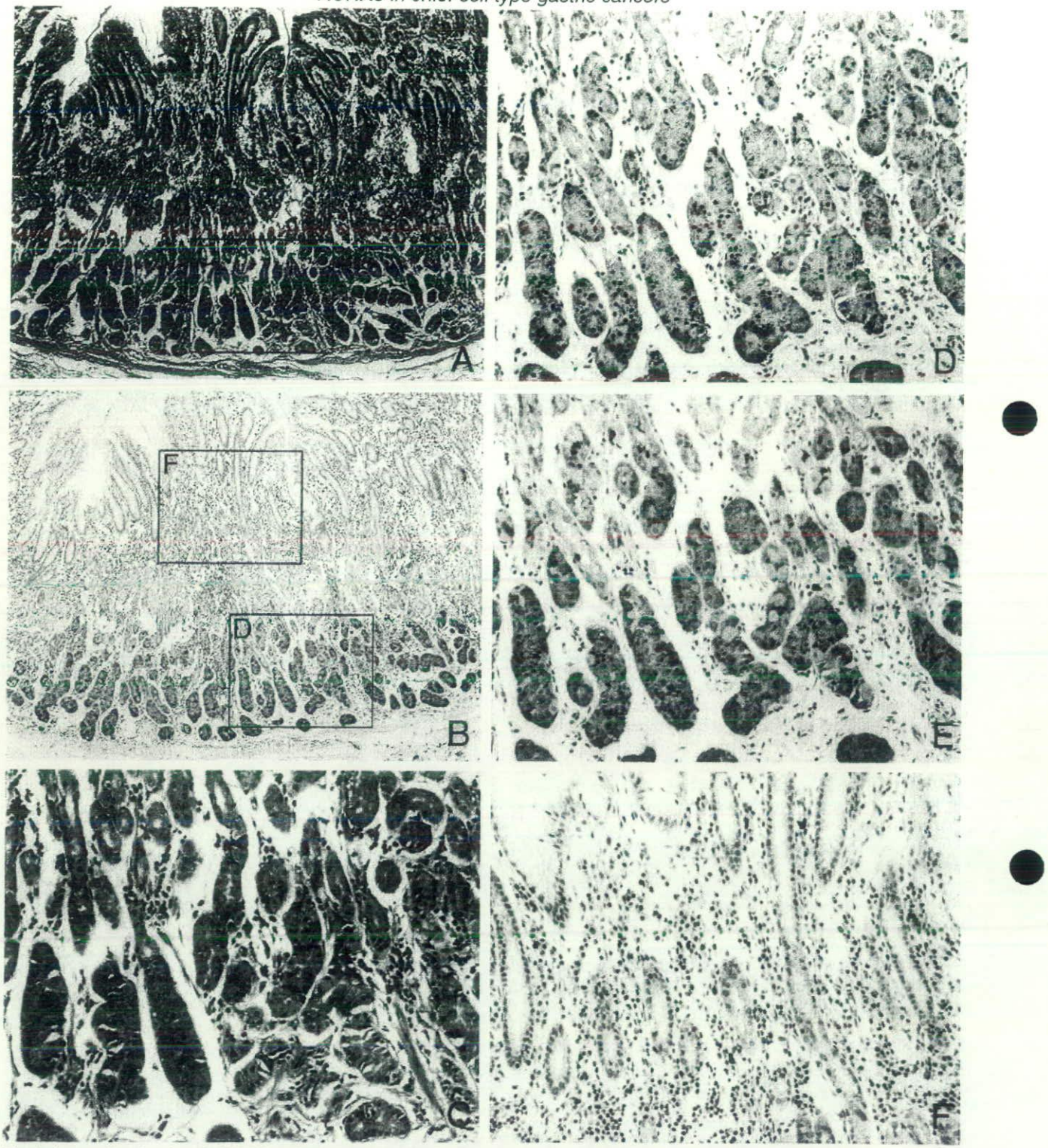

Fig. 1. Immunohistochemistry for RUNX3 and pepsinogen I in the fundic gland area of normal gastric mucosa. RUNX3 expression is apparent in the pepsinogen I-expressing chief cells in fundic glands. **A,** H&E staining; **B,** RUNX3 staining, lower magnification; **C,** higher magnification of A, showing basophilic chief cells and acidophilic parietal cells in fundic glands; **D,** RUNX3 staining, higher magnification of B, RUNX3 is positive in the chief cells, but not in the parietal cells of normal gastric mucosa; **E,** Pepsinogen I staining, higher magnification of the area in D, Pepsinogen I is expressed in the fundic chief cells expressing RUNX3; **F,** RUNX3 is weakly stained in the cytoplasm of normal gastric foveolar epithelium, as well as monocytes and lymphocytes in the stromal tissue of the normal gastric mucosa. Original magnification; A and B, x 50; C-E, x 200; F, x 100

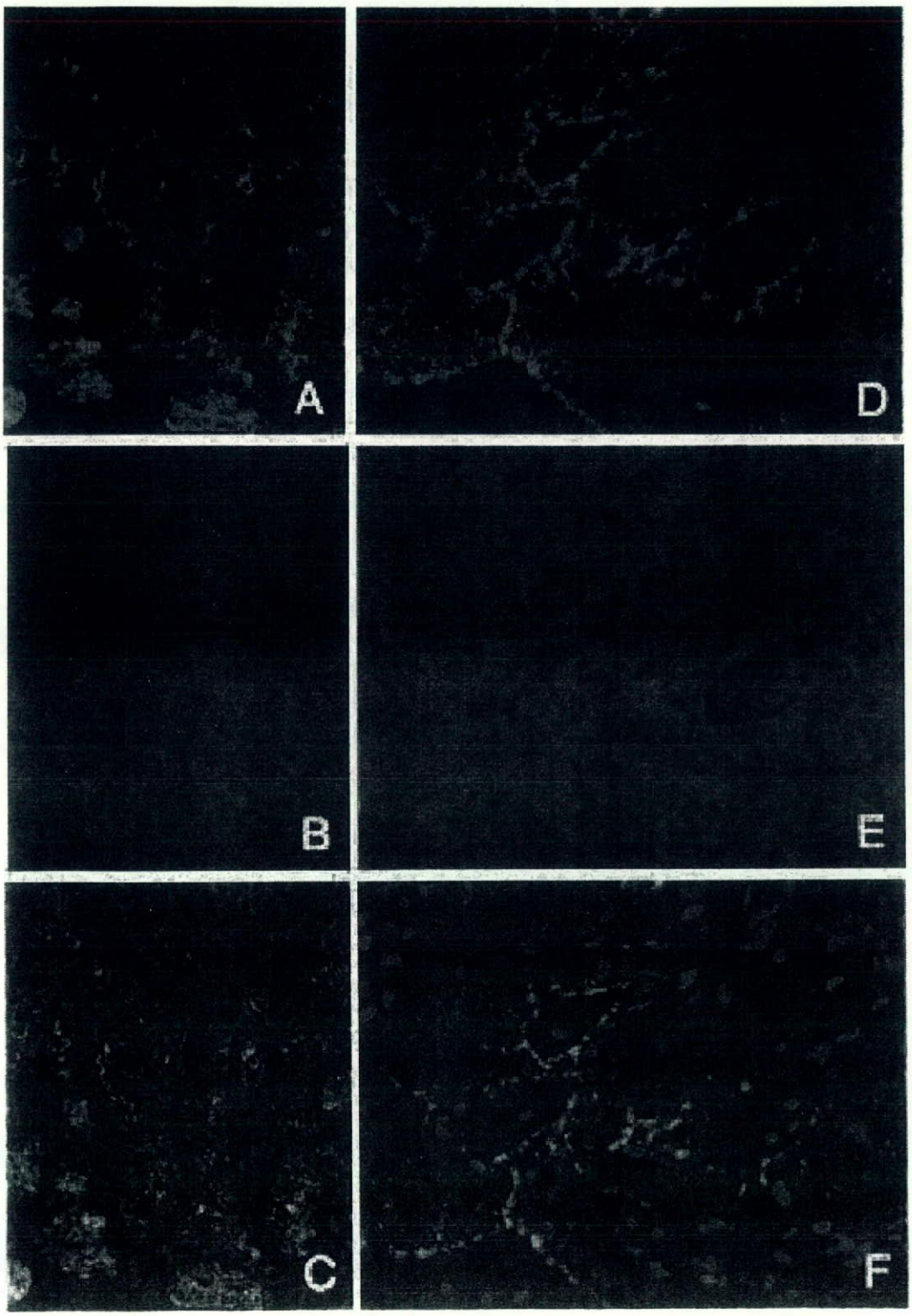

Fig. 2. Coexpression of pepsinogen I and RUNX3 in normal fundic mucosa and gastric cancers. **A-C.** Bottom part of normal fundic mucosa. **D-F.** Gastric adenocarcinoma. Expression of pepsinogen I (**A and D**, FITC) and RUNX3 (**B and E**, rhodamine). Merged figures are shown with nuclear counter staining (**C and F**, DAPI). Original magnification: A-C, x 200; D-F, x 400

negative cancers were 37% and 46%, respectively, the difference again not being significant.

No relationship between RUNX3 expression and intestinal phenotypic expression

62 and 40 cases were judged to be MUC2, villin, and/or Cdx2 positive and negative (Table 3). The rates for RUNX3 positive cases were 42% and 48%,

Table 2. The relation between RUNX3 expression and the morphological classification.

	RUNX3*		Total
	+	−	
Differentiated adenocarcinomas	24 (45%)	29 (55%)	53
Undifferentiated adenocarcinomas	21 (43%)	28 (57%)	49
Total	45	57	102

*P=0.844

Table 3. The relation between RUNX3 expression and the phenotypic markers.

Antigens	Target cells		RUNX3		Total	P values
			+	−		
MUC5AC	Gastric foveolar (Surface mucous) cell	+	21 (41%)	30 (59%)	51 (100%)	P=0.69
		−	24 (47%)	27 (53%)	51 (100%)	
MUC6	Pyloric gland and mucous neck cell	+	7 (37%)	12 (67%)	19 (100%)	P=0.61
		−	38 (46%)	45 (54%)	83 (100%)	
Pepsinogen I	Chief cell	+	26 (79%)	7 (21%)	33 (100%)	P<0.001
		−	20 (26%)	49 (74%)	69 (100%)	
Proton pump	Parietal cell	+	0 (0%)	0 (0%)	0 (100%)	P>0.99
		−	45 (44%)	57 (56%)	102 (100%)	
MUC2, villin, and/or Cdx2	Intestinal metaplastic cell	+	26 (42%)	36 (58%)	62 (100%)	P=0.68
		−	19 (48%)	21 (52%)	40 (100%)	

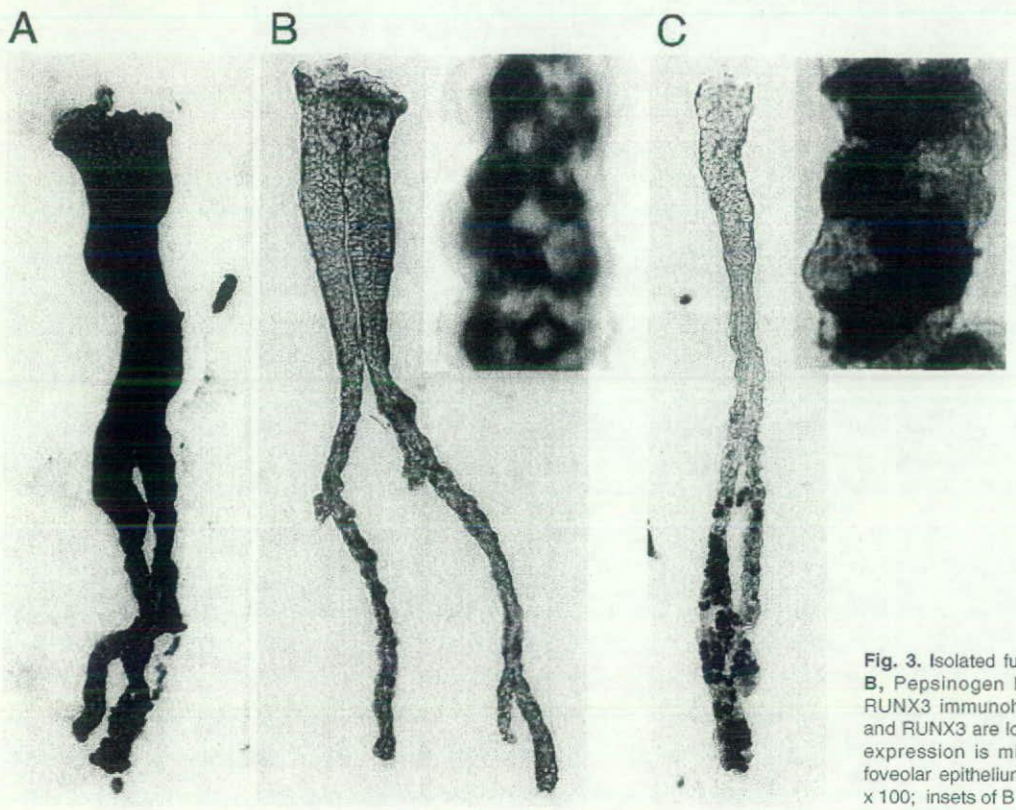

Fig. 3. Isolated fundic glands. **A,** H&E staining; **B,** Pepsinogen I immunohistochemistry; **C,** RUNX3 immunohistochemistry. Pepsinogen I and RUNX3 are localized in the basal region but expression is minimal in the upper parts of foveolar epithelium. Original magnification: A-C, x 100; insets of B and C, x 640

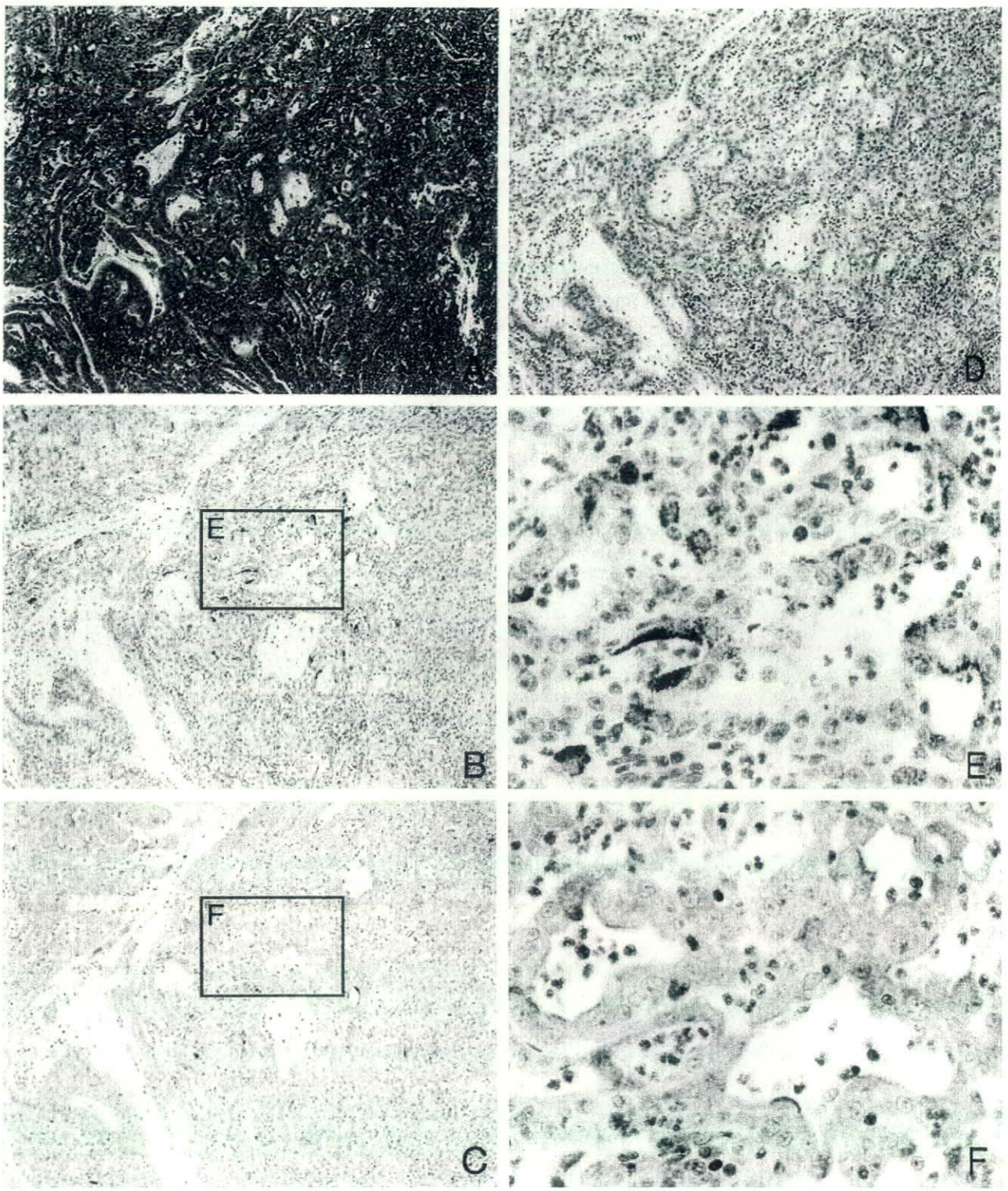

Fig. 4. Immunohistochemistry for RUNX3, pepsinogen I, and the proton pump in gastric cancer. Gastric cancer cells with pepsinogen I cytoplasmic staining also express RUNX3, with an almost perfect overlap. **A,** H&E staining; **B and E**, pepsinogen I staining; **C and F**, RUNX3 staining; **D,** Proton pump staining; **E,** higher magnification of B; **F,** higher magnification of C. Original magnification: A-C, x 100; D and E, x 400

respectively, the difference not being significant.

No relationship between RUNX3 expression and Proton pump expression

Of the 102 gastric cancers, all cases were judged as proton pump expression negative, indicating no relevance to parietal cells (Table 3).

RUNX3 mRNA and pepsinogen I mRNA expressions in the gastric cancer cell line

RUNX3 and pepsinogen I expression were revealed in MKN45 human gastric carcinoma cell line after 35 cycles of PCR reaction. In other cell lines, however, only pepsinogen I was detectable in MKN74 and no visible bands were obtained in TMK1 (Fig. 5). Thus, clear correlation was not assumed between transcriptional expression of RUNX3 and pepsinogen I.

Discussion

In the present study, RUNX3 expression was determined immunohistochemically in 102 gastric carcinomas for the purpose of determining interrelations among their RUNX3, pepsinogen I, proton pump, MU5AC, MUC6, and intestinal phenotypic expression.

It was reported recently that loss of expression of RUNX3 is causally related to the genesis and progression of gastric cancer (Li et al., 2002; Ito et al., 2005). About 45% to 60% of surgically resected gastric cancer specimens and associated cell lines do not express RUNX3 due to hemizygous deletion of the gene or hypermethylation of its promoter region (Li et al., 2002). Inactivation of RUNX3 appears to occur at an early stage, as well as during progression, because silencing of RUNX3 has been observed in 40% of stage I and 90% of stage IV gastric cancers (Li et al., 2002). A mutation found in a gastric cancer patient, RUNX3 (R122C), which causes a single amino acid substitution within the conserved DNA-binding domain, completely abolished the tumor suppressor activity of RUNX3, as assessed in a nude mouse assay. Hyperplasia of the gastric epithelium, observed in the Runx3(-/-) experimental mouse system, seems to be caused by decreased sensitivity to TGF-ß, which inhibits cell cycle progression and induces apoptosis. Furthermore, experiments with stomach epithelial cell lines isolated from Runx3(+/+) and Runx3(-/-) mice with the p53(-/-) background revealed that only those lines derived from Runx3(-/-) p53(-/-) mice were tumorigenic in nude mice (Li et al., 2002). Although these results strongly suggest that RUNX3 is a gastric cancer tumor suppressor and that its loss is involved in roughly half of the cases of gastric cancer, we assumed that it functions normally in the remaining cases (Ito et al., 2005). RUNX3 was here strongly expressed in chief cells in fundic glands, but only to a limited extent in parietal cells in the deeper zone, and was not detectable in cells in the generative zone, suggesting that RUNX3 may play a specific functional role in chief cells (Ito et al., 2005; Osaki et al., 2004). Li et al. reported gastric mucosa to be hyperplastic in RUNX3-deficient mice, leading us to speculate that RUNX3 may be implicated in growth control of chief cells (Li et al., 2002). Further analysis is required to address this possibility.

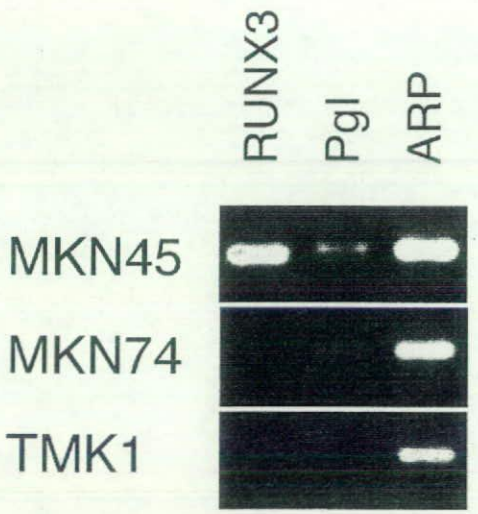

Fig. 5. Transcriptional expression of RUNX3 and pepsinogen I (PgI) in gastric cancer cell lines. Acidic ribosomal phosphoprotein PO (ARP) as an internal control

It is widely thought that the phenotypic expression of tumor cells resembles that of the tissue of origin. Further, it has been shown that gastric cancers at early stages, independent of the histological type, mainly consist of gastric phenotypic cancer cells. A shift from gastric to intestinal phenotypic expression then clearly occurs with progression (Tatematsu et al., 1990, 2003; Yamachika et al., 1997; Yoshikawa et al., 1998; Bamba et al., 2001). The present study showed, for the first time to our knowledge, a relation between RUNX3 and pepsinogen I expression in human gastric cancers, although there have been reports regarding pepsinogen I in gastric cancers (Stemmermann et al., 1985; Huang et al., 1988). Thus the present data provide clear evidence that the RUNX3 is associated with the differentiation of chief cells in cancers, as well as normal gastric mucosa. We also recently experienced a rare case of gastric adenocarcinoma with chief cell differentiation harboring strong pepsinogens I and II, MUC6, and RUNX3 expression characteristic of primitive chief cells (Tsukamoto et al., 2007). Links to gastric foveolar epithelial cells have also been hypothesized (Li et al., 2002; Osaki et al., 2004), but there was no relationship between RUNX3 and MUC5AC or other differentiation markers of gastric epithelial cells in the present study. However, expression analysis of RUNX3 and pepsinogen I using stomach cancer cell lines revealed

ambiguous correlation between these factors. MKN45 was shown to have significant amount of RUNX3 and pepsinogen I, compatible with the results obtained that in the immunohistochemistry. Nonetheless, MKN74 possessed only pepsinogen I transcript and TMK1 did not harbor both of them. These results suggested that pepsinogen I could not be regulated under RUNX3 but might be coexpressed in the chief cells in the fundic gland.

In conclusion, our data suggest that RUNX3 is strongly associated with chief cell phenotypic expression in human gastric cancers, as well as the human normal gastric mucosa. RUNX3 may have essential roles in cell differentiation in normal fundic glands and gastric cancers.

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Expression of osteopontin and CDX2: Indications of phenotypes and prognosis in advanced gastric cancer

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Abstract. We have investigated the expression of osteopontin (OPN) and CDX2 in advanced gastric cancers, and analyzed correlations with clinicopathological features to assess their prognostic potential. One-hundred and nine patients suffering from gastric cancer were recruited. Expression of OPN and CDX2 and other molecular markers was determined by immunohistochemistry. The total positive rate for OPN expression was 46.8%, with a relation to depth of cancer invasion and down regulation of intestinal markers (P<0.001), but not age, gender, or histological type. OPN was more frequently expressed in CDX2-negative (39/109=35.7%) as compared with positive lesions (12/109=11.0%) and a significant reverse correlation was noted between the two factors (P<0.001). Patients with positive OPN tumors had worse 5-year survival than those with OPN-negative cancer (P<0.001). Further analysis revealed the $OPN^-/CDX2^+$ group to have better 5-year survival than all the other three groups: $OPN^+/CDX2^-$, $OPN^-/CDX2^-$ and $OPN^+/CDX2^+$. With multivariate analysis for 5-year survival, OPN was the most significant predictor of a poor prognosis of advanced gastric cancer (P=0.0043), with tumor depth of invasion as another independent indicator (P=0.0315). Osteopontin is a useful prognostic marker in gastric cancer, and combined with CDX2, may have particular advantage for predicting survival of advanced gastric cancer patients. Furthermore the present results provide a clue that in gastric cancer, CDX2 may be a transcription factor modulating the expression of osteopontin.

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Key words: osteopontin, CDX2, gastric cancer, phenotype, prognosis

Introduction

Gastric cancer (GC) is one of the most aggressive tumors and the second leading cause of cancer mortality worldwide (1). Finding a useful molecular marker to predict malignant potential is therefore of great importance. Although many molecular indicators have been reported (2,3), there are still problems with accurate prediction. Osteopontin (OPN), a 34 kDa extracellular matrix glycophosphoprotein with a cell-binding domain, plays multifunctional roles in cell adhesion, chemotaxis, macrophage-dependent angiogenesis, prevention of apoptosis, and anchorage-independent growth of tumor cells. Its activity regulates cell-matrix interactions and cellular signaling through binding to integrin and CD44 receptors (4-6). It has been widely reported to demonstrate altered expression in relation to tumorigenesis, invasion, metastasis and its expression may have prognostic potential in colon (7), lung (8), prostate (9), and breast cancers (10). Overexpression correlated with poor prognosis in gastric cancer has also been reported (11,12).

In previous studies, others and our group have demonstrated that expression of the caudal-related homeobox gene (CDX) 2 is strongly associated with an intestinal phenotype in gastric cancer (13,14), providing a useful prognostic marker for intestinal and gastrointestinal phenotypic gastric tumors with good outcomes (15,16). To further probe useful indicators for gastric cancer survival, in the present retrospective study we examined the expression of OPN and CDX2 in 109 advanced gastric cancer surgical specimens, and analyzed correlations with clinicopathological factors. A particular focus was on links between OPN and CDX2 expression and the different phenotypes of gastric cancer.

Materials and methods

Patients and tumor specimens. All 109 cases of primary advanced gastric cancer were surgically resected at Aichi Cancer Center Hospital, Nagoya, Japan, between 1994 and 1996 after obtaining informed consent. The patients were 63 males and 46 females and the mean age was 62.43±10.12 years.

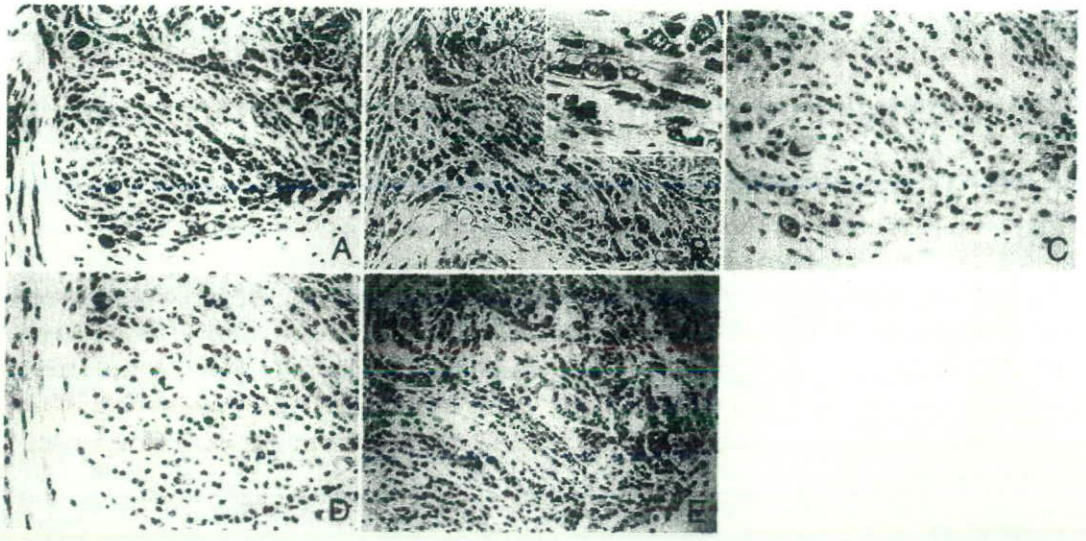

Figure 1. Poorly differentiated gastric adenocarcinomas. (A) H&E staining. (B-E) Immunohistochemical analysis. Osteopontin (B) is present in the cytoplasm, CDX2 (C) and MUC2 (D) being barely detected in the same area. MUC5AC (E) is weakly positive. Original magnification, x200. Inset of B, x400.

None had received preoperative chemotherapy or radiotherapy before surgery. All specimens were fixed in 10% buffered formalin. Carcinomas with adjacent mucosa tissue were serially cut into 3-mm slices and embedded in paraffin, and then thin-sectioned and stained with hematoxylin and eosin for histological examination.

Immunohistochemistry. We examined expression of MUC5AC, MUC6, MUC2, and villin in carcinoma cells by immunohistochemistry, as previously described in detail (17,18). Briefly, 3 μm-thick consecutive sections were deparaffinized and hydrated through a graded series of ethanols. After inhibition of endogenous peroxidase activity by immersion in 3% H_2O_2/methanol solution, antigen retrieval was carried out with 10 mM citrate buffer (pH 6.0) in a microwave oven for 10 min at 98°C. Then, sections were incubated with the primary antibodies. After thorough washing in phosphate-buffered saline (PBS), incubation with biotinylated secondary antibody was performed, followed by exposure to avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA). Finally, immune-complexes were visualized by incubation with 0.01% H_2O_2 and 0.05% 3,3'-diaminobenzidine tetrachloride (DAB). Nuclear counterstaining was accomplished with Mayer's hematoxylin. We also examined expression of CDX2 using an anti-CDX2 monoclonal antibody (BioGenex, San Ramon, CA, USA) and expression of osteopontin with anti-osteopontin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using the same immunohistochemical approach. The results of antibody staining were evaluated with reference to the percentage of positively stained cancer cells. A result was considered positive if at least 10% of the cells were stained. The results were evaluated by two of the authors (X.Z. and T.T.) without any previous knowledge of the clinical information for each patient.

Classification of phenotypes. As we previously reported, MUC5AC and MUC6 are markers of gastric epithelial cells, whereas MUC2 and villin are typical of the intestinal epithelial cell phenotype (19,20). In gastric cancers, if >10% of the section area expresses at least one of the markers specific for gastric or intestinal phenotypes classification is made as gastric (G type) or intestinal (I type), respectively. Those which show both gastric and intestinal phenotypes are classified as gastric-and-intestinal-mixed phenotype (GI type) cancers, while those showing neither expression are grouped as null type (N type).

Tumor staging. Classification of tumor staging was made according to the Japanese Classification of Gastric Carcinomas (21). The cancers had invaded the muscularis propria (T2 for TNM classification), the subserosa (T2), or the serosa and the peritoneal cavity (T3), sometimes including the adjacent organs (T4).

Statistical analysis. The data were analyzed by Fischer's exact test or χ^2-test for differences between groups using StatView statistical software (ver. 5, SAS Institute, Inc., Cary, NC, USA). To determine the relative survival of patients, the Cox's proportional-hazards regression model was used, and survival curves after surgery were drawn using the Kaplan-Meier method. Statistical comparison of survival was performed using the log-rank test. $P<0.05$ were considered statistically significant.

Results

OPN expression in GC tissues and correlation with clinicopathological factors. The follow-up period of the patients ranged from 4 to 96 months. Among all 109 cases, the positive OPN immunohistochemistry expression rate was 46.8% (51/109) (Table I). OPN was frequently expressed in the cytoplasm of gastric tumor cells (Fig. 1) and commonly found to be most intense in the margins of tumor tissue or in tumor cells invading into the muscle layer or serosa. OPN expression was significantly related with tumor depth

Table I. OPN expression and clinicopathological factors.

Clinicopathological data	n=109	OPN expression		P-values
		Positive (n=51)	Negative (n=58)	
Gender				
Male	63	21	42	NS
Female	46	17	26	
Age				
Years (means ± SD)		63.34±10.17	61.78±11.96	NS
Histological classification				
Differentiated	58	43	15	NS
Undifferentiated	51	36	15	
Phenotypes				
G	18	16	2	P<0.001
GI	19	7	12	
I	40	7	33	
N	32	21	11	
Cdx2				
Positive	57	12	45	P<0.001
Negative	52	39	13	
Depth of invasion				
T1	31	4	27	P<0.001
T2-T3	52	21	21	
T4	36	26	10	

(P<0.001), positive rates being 12.9, 50.0 and 72.2% for T1, T2-T3, and T4, respectively (P<0.001). However, OPN expression did not correlate with patients' age, gender, or histological status (Table I).

Expression of gastric and intestinal epithelial cell markers in gastric cancers. Expression of MUC5AC, MUC6, MUC2, and villin were judged positive in 54 (49.5%), 29 (26.6%), 51 (48.1%), and 47 (43.1%) cases, respectively. Taking into account the combinations of expression of these four markers, the 109 gastric cancers were divided phenotypically into 18 G, 19 GI, 40 I, and 32 N types, independent of the histological classification (Fig. 1 and Table I).

OPN expression in different phenotypes of GC and its correlation with CDX2. OPN was mainly expressed in G and N type (16 and 21 cases, respectively) and less expressed in GI and I type (7 cases each) (P<0.001) (Table I). Based on these results and our previously published data (15) for advanced GC with intestinal phenotypic expression, CDX2 is a useful marker of a good prognosis. Further analysis of the correlation of OPN and CDX2 expression showed only 12 of 51 OPN-positive to be CDX2 expression-positive. However, of 58 OPN-negative cases, 45 demonstrated binding of the CDX-2 antibody. Thus an inverse correlation was observed (P<0.001).

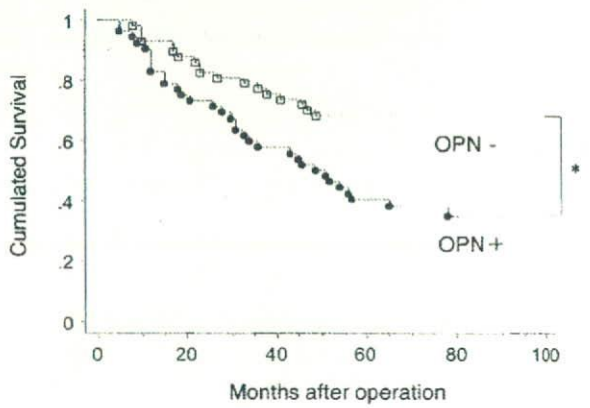

Figure 2. Kaplan-Meier cumulated survival curves for the 109 GC patients with reference to OPN expression. *P<0.001.

In almost all GCs, areas with OPN-positive expression were CDX2-negative (Fig. 1 and Table I).

Postoperative survival analysis of GCs with reference to OPN and CDX2 expression. Among the 109 cases of GC, the 5-year survival rates with OPN-negative and -positive lesions were 77.6 and 25.5%, respectively. From the Kaplan-Meier

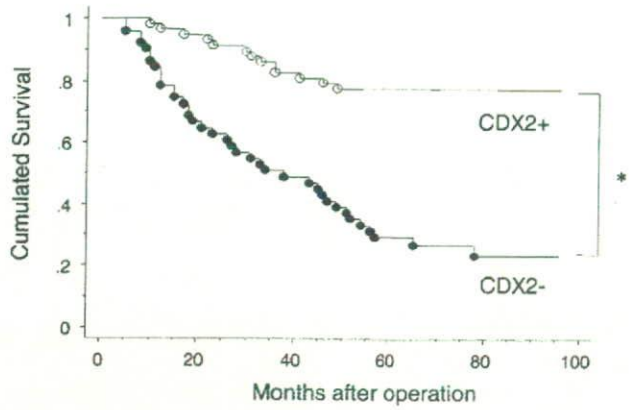

Figure 3. Kaplan-Meier cumulated survival curves for the 109 GC patients with reference to CDX2 expression. *P<0.001.

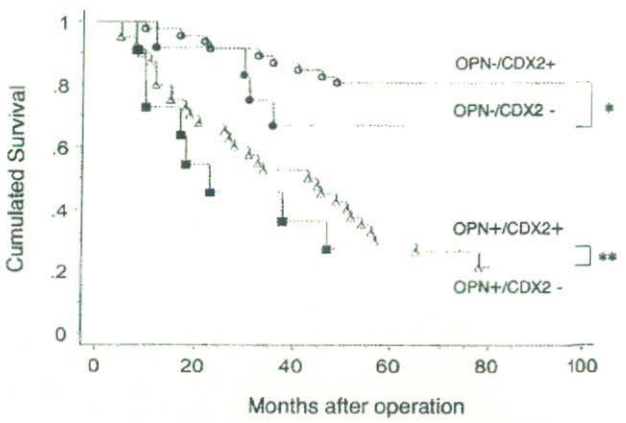

Figure 4. Kaplan-Meier cumulated survival curves for the 109 GC patients with reference to OPN and CDX2 expression. *P=0.013 ($OPN^-/CDX2^+$ vs. $OPN^-/CDX2^-$); **P=0.093 ($OPN^+/CDX2^+$ vs. $OPN^+/CDX2^+$).

survival curve analysis, the patients with OPN-negative expression had the better overall survival (P<0.001) (Fig. 2). Fig. 3 shows that the 5-year survival rates with CDX2-positive and -negative expression were 68.4 and 36.5%, respectively (Kaplan-Meier survival curve analysis, P=0.0025). Furthermore, 5-year survival rates for $OPN^-/CDX2^+$, $OPN^-/CDX2^-$, $OPN^+/CDX2^+$, and $OPN^+/CDX2^-$ were 80.4, 66.7, 27.2 and 25.2%, respectively (Fig. 4). The patients with $OPN^-/CDX2^+$ cancers had a better 5-year survival outcome than the other three groups (P<0.01). The 5-year survival of the $OPN^-/CDX2^-$ group was better than that of the $CDX2^-$ (P=0.024) or $CDX2^+$ group (P=0.028) with OPN^+ expression. However, in $OPN^+/CDX2^-$ and $OPN^+/CDX2^+$ groups, the difference in 5-year survival was not significant (P=0.093) (Fig. 4).

Multivariate analysis for overall survival of GC cases. Using the Cox' proportional hazards regression model, we performed multivariate analysis of clinicopathological variables, including the patient age, gender, tumor histological classification, phenotypic classification, tumor depth, and OPN and CDX2 expression. This revealed OPN to be the most independent factor for 5-year overall survival (P=0.0043). Tumor depth of invasion was also an independent indicator (P=0.0315). CDX2 expression status, patient age, gender, phenotypic type, and histological status were not independent factors for overall survival of gastric cancer cases (P>0.05).

Discussion

OPN is a highly modified integrin-binding extracellular matrix glycophosphoprotein produced by cells of the immune system, epithelial tissue, smooth muscle cells, osteoblasts, and tumor cells. Although extensive research has elucidated pivotal roles of OPN in cell signaling relevant to inflammation, tumor progression and metastasis, and inhibition of apoptosis, the mechanisms by which OPN may enhance malignancy in gastric cancer are still unclear. Clearly, molecular binding to the cell adhesion molecules integrin and CD44 (4,22) and depletion of growth factors and cytokines (23) could be involved. Of prime importance, however, is the link with prognosis revealed by the present study, in line with the association with malignancy reported earlier (5,24,25).

CDX2 is a caudal-related homeobox transcription factor that is expressed specifically in intestine epithelial cells (15,26,27), playing a probable role in regulation of their proliferation. It is well known that both gastric and intestinal phenotypic cell markers are expressed in gastric cancers and CDX2 expression is evident in a high proportion of early intestinal-type cancers, becoming reduced with perineural invasion and lymph node metastasis. Thus CDX2 might be a useful marker in predicting the clinical outcome for patients with gastric cancers (28-30). In Mongolian gerbils, celecoxib, a cyclooxygenase-2 inhibitor, suppressed the expression of CDX2 and prevented intestinal metaplasia and gastric carcinogenesis (31). In our previous study, we also established that in mixed GI and I phenotypes of gastric cancer with CDX2 expression, the prognosis was significantly better than with G or N types harboring little CDX2 (15). We also found that in GI and I phenotypes with high CDX2 expression, the OPN expression was low, whereas in G and N types without CDX2, the OPN expression level was high.

Multivariate analysis revealed OPN as the most independent factor for survival, followed by tumor depth and lymph node metastasis. In contrast, the patient gender, age, tumor histological type, and CDX2 expression status were not independent prognostic factors. In conclusion, OPN is a very useful indicator for predicting the prognosis of gastric cancer, and combined with CDX2, might give a particularly accurate picture.

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Regular Paper

Gene Expression Profiling Distinguishes Between Spontaneous and Radiation-induced Rat Mammary Carcinomas

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Ionizing radiation/Breast cancer/Sprague-Dawley rats/Radiation signature/Expression microarray.

The ability to distinguish between spontaneous and radiation-induced cancers in humans is expected to improve the resolution of estimated risk from low dose radiation. Mammary carcinomas were obtained from Sprague-Dawley rats that were either untreated (n = 45) or acutely γ-irradiated (1 Gy; n = 20) at seven weeks of age. Gene expression profiles of three spontaneous and four radiation-induced carcinomas, as well as those of normal mammary glands, were analyzed by microarrays. Differential expression of identified genes of interest was then verified by quantitative polymerase chain reaction (qPCR). Cluster analysis of global gene expression suggested that spontaneous carcinomas were distinguished from a heterogeneous population of radiation-induced carcinomas, though most gene expressions were common. We identified 50 genes that had different expression levels between spontaneous and radiogenic carcinomas. We then selected 18 genes for confirmation of the microarray data by qPCR analysis and obtained the following results: high expression of *Plg*, *Pgr* and *Wnt4* was characteristic to all spontaneous carcinomas; *Tnfsf11*, *Fgf10*, *Agtr1a*, *S100A9* and *Pou3f3* showed high expression in a subset of radiation-induced carcinomas; and increased *Gp2*, *Areg* and *Igf2* expression, as well as decreased expression of *Ca3* and non-coding RNA *Mg1*, were common to all carcinomas. Thus, gene expression analysis distinguished between spontaneous and radiogenic carcinomas, suggesting possible differences in their carcinogenic mechanism.

INTRODUCTION

Cancers arise from various types of cells via multiple oncogenic pathways that may involve various genetic and epigenetic alterations. Ionizing radiation is a well-known cause of human cancers. Humans are constantly exposed to cosmic radiation and naturally occurring radioactivity (*e.g.*, from radon gas and its decay products), but the largest component of radiation exposure is from medical sources.[1)] Whereas increased risks of cancers from exposure to large doses are unquestionable, the risk estimates at low doses are difficult because the estimated excess risk is much smaller than the background risk, which can be easily confounded by other factors.[2)] Currently, low dose radiation exposure risks are estimated by extrapolating high-dose risks, albeit with large uncertainty. Thus, it is anticipated that, if molecular fingerprints of radiation-related cancer could be established, it would help improve the risk estimations at low doses.[3)] However, investigation of genetic changes in cancer-related genes have not produced information on radiation-associated alterations with only a few exceptions.[4–6)] Recent evidence indicates that radiation induces persistent genetic instability in the progeny of irradiated cells, and the spectrum of these resulting mutations is very similar to that of spontaneously arising mutations, which implies that radiation increases the rate of spontaneous cancer incidence by enhancing accumulation of mutations.[7–9)] Alternatively, it is hypothesized that the carcinogenic effect of radiation is mediated by induction of clonal expansion of cells which already harbor spontaneously-arising mutations of cancer-related genes.[10)]

Analysis of gene expression profiles for tumors is a powerful tool in cancer biology. It has been utilized to classify cancer subtypes, to predict therapeutic outcomes and to choose appropriate targeted therapies.[11–13)] Recently, rat mammary cancers induced by several chemical carcinogens have been compared based on their gene expression profiles,

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and the profiles of these cancers reflect, to some extent, differences among etiological agents.[14–16] Rat mammary cancer is an important experimental model of human breast cancer due to similarities in both pathology and hormone dependence.[17,18] Several epidemiological studies have shown that breast cancer is one of the most prevalent cancers after radiation exposure.[19,20] Thus, the rat model of mammary cancer has been a useful tool to analyze radiation-induced breast cancer.[21–23] Animal cancer models are also advantageous because they are induced by defined carcinogenic agents in contrast to human cancers, which are rarely ascribed to a single etiological factor.

Thus, comparison of gene expression profiles should provide evidence of molecular characteristics that distinguish between radiation-induced and spontaneously developed rat mammary carcinomas. In the present study, we analyzed gene expression profiles of mammary carcinomas from irradiated and non-irradiated rats using oligonucleotide microarrays in combination with quantitative polymerase chain reaction (qPCR) and showed that ionizing radiation–induced and spontaneous cancers can be distinguished based on their gene expression profiles.

MATERIALS AND METHODS

Mammary carcinomas and normal mammary glands

Mammary carcinomas were collected in our previous study.[24,25] Briefly, 7-week-old Sprague Dawley rats were either treated with γ rays (1 Gy, $n = 20$) or left untreated ($n = 45$). All rats were fed a high corn-oil diet, and rats with palpable tumors were sacrificed at 1 year of age, or earlier in case of moribundity, for tissue collection. As reported previously, the multiplicities of palpable mammary carcinoma that developed in untreated and irradiated rats before one year of age were 0.067 and 0.506, respectively.[25] We refer to them as spontaneous and radiation-induced carcinomas, hereafter. Their first ages of detection were 32.0 ± 5.9 and 29.6 ± 11.2 weeks of age (mean ± standard deviation), respectively, and were not significantly different.[25] For molecular analyses, we added one spontaneous carcinoma that developed later at 70 weeks of age and used in total 4 spontaneous and 10 radiation-induced adenocarcinomas that were histologically uniform (papillotubular and tubular types) and contained no identifiable necrotic region. Normal mammary tissues were collected from rats of the untreated group (1 year of age) that did not develop mammary carcinoma. Tissues were snap-frozen in liquid nitrogen and stored at –80°C until use.

Microarray analysis

Three spontaneous and four radiation-induced carcinomas, as well as three normal mammary glands, were randomly selected for microarray analysis. GeneChip Rat Genome 230 2.0 arrays carrying 31,024 probe sets, where each probe set corresponds to one gene sequence, were purchased from Affymetrix (Santa Clara, CA, USA). The procedures for complementary RNA (cRNA) labeling, hybridization and image scanning were essentially identical to those described.[14] Briefly, total RNA was isolated by the acid guanidine phenol chloroform method (Isogen; Nippon Gene, Tokyo, Japan) and further purified with a silica-gel membrane (RNeasy Mini kit; Qiagen Inc., Valencia, CA, USA). The quality of all RNA samples was assessed by formalin-containing agarose gel electrophoresis. Total RNA (8 μg) was used for the first-strand cDNA synthesis with a T7-$(dT)_{24}$ primer (Proligo, Kyoto, Japan) and SuperScript III reverse transcriptase (Invitrogen Co., Carlsbad, CA, USA). Double-stranded cDNA was then synthesized with *E. coli* RNase, *E. coli* DNA polymerase and *E. coli* DNA ligase (Toyobo, Tokyo, Japan). Biotin-labeled fragmented cRNA was subsequently prepared with a BioArray HighYield RNA Transcript Labeling kit (Enzo, Farmingdale, NY, USA). Labeling was confirmed by formalin-containing agarose gel electrophoresis. Labeled cRNA was placed in a hybridization mixture containing control biotinylated probes according to manufacturer's instructions. GeneChip arrays were hybridized with labeled cRNA for 16 h at 45°C with constant rotation (60 rpm). The arrays were washed and then stained with streptavidin-phycoerythrin conjugate (Molecular Probes, Tokyo, Japan) in a Fluidics Station 450 (Affymetrix) and subsequently scanned with the GeneChip Scanner 3000 (Affymetrix). The scanned images were processed using Affymetrix GeneChip Analysis Suite software. Each data set was scaled such that the average intensity of all probe sets was adjusted to 500. Data were exported to flat text files and used for statistical analysis.

Data analysis

Probe sets with fluorescent intensity values less than 1,000 for all carcinoma arrays were excluded in order to confine analysis to quantitatively reliable data. Before conducting clustering analysis, the fold change values, in comparison with the average intensity values for normal mammary glands, were transformed to base-2 logarithms. Average linkage clustering of an uncentered Pearson correlation similarity matrix was applied with the Cluster software, and the figures were generated with the TreeView program.[26] Similarities among expression profiles were assessed by the Pearson correlation coefficient.[26] Welch's *t*-test was used to calculate *P* values for gene selection.

qPCR analysis

First-strand cDNA was synthesized from purified total RNA as described.[27] The qPCR reaction was performed on an Mx3000P real-time PCR system (Stratagene, La Jolla, CA, USA). The expression of a housekeeping gene, *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase), was first measured as an internal standard by qPCR (TaqMan Rodent

Table 1. Primers and their annealing temperatures for quantitative PCR

Gene symbol [a]	Gene name	Forward primer Reverse primer [b]	T_a [c]
Plg	Plasminogen	TGTGCAACCGCGCTGAGTAT	60
		AGCACAGCCAAGACCCCAAG	
Rn.160502	EST	AGGAGGGCCCAGAGTCCAAG	60
		AGGCGAGACAGCGAGAAGGA	
Pgr	Progesterone receptor	GGGTGGTCCCCAGTTCACAA	60
		CCGGAAATTCCACAGCCAGT	
Wnt4	Wingless-related MMTV integration site 4	ACAACGAGGCTGGCAGGAAG	60
		TTAGTGCGTGGCCAACCTGA	
Rn.20273	EST	GGTTCCAGCACGTTGGTCCT	60
		TGTAATCGTTCTCCTCTTGGGACA	
Kit	v-kit oncogene homolog	TGCCGGTCGATTCCAAGTTT	60
		TTGGCCTTTTCAGGGGATCA	
Tnfsf11	Tumor necrosis factor superfamily member 11	GGAAGGTTCGTGGCTCGATG	60
		GCCCAGCCTCGATCATGGTA	
Fgf10	Fibroblast growth factor 10	GGGAGATGTCCGCTGGAGAA	60
		CGGCAACAACTCCGATTTCC	
Agtr1a	Angiotensin II receptor type 1	TGGCTGGCATTTTGTCTGGA	60
		CCTTGGGGCAGTCATCTTGG	
Pou3f3	POU domain class 3 transcription factor 3	GGCGCAGGAGATCACCAACT	60
		GGTCCCCACCTGCGAGTAGA	
S100a9	S100 calcium binding protein A9	TGGACATCCTGACACCCTGAA	64
		GGTTTGTGTCCAGGTCCTCCA	
Rn.177404	EST	CCTCCCAGGCTTTCCCACTT	60
		GAGTGCCACCGGATCTTTGG	
Ptges	Prostaglandin E synthase	ACGCGTTGAAACGTGGAGGT	60
		AGAGGGTTGGGTCCCAGGAA	
Gp2	Glycoprotein 2	TCGCAGTAGTGAACCAGCCATC	60
		GCCAGGAAGACAGGCAGGAA	
Areg	Amphiregulin	CGTCGCAGCTATTGGCATCA	60
		TGGCTTGGCAGTGACTCGAC	
Igf2	Insulin-like growth factor 2	GGACCGCGGCTTCTACTTCA	60
		CACGTCCCTCTCGGACTTGG	
Ca3	Carbonic anhydrase 3	GGACGGGAGAAAGGCGAGTT	60
		CCAATAGTCCCGGCAAGCAG	
RGD:727910	Mg1 protein	CAGTGCTGCCAAGACCCTGA	60
		CCACCATCCCTCACACTCACA	

[a] The Unigene ID is shown for unidentified expressed sequence tags (ESTs).
[b] Base sequences are indicated in the order of 5' to 3'.
[c] Annealing temperature (°C).

GAPDH Control Reagent, VIC Probe; Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The concentration of cDNA in samples was adjusted so that the fluctuation of *Gapdh* expression was less than two-fold between samples. Then qPCR analysis on genes of interest was performed with a commercial mixture of Taq DNA polymerase and a fluorescent dye (SYBR Premix Ex Taq; Takara Bio Inc., Otsu, Japan). The PCR program consisted of denaturation at 95°C for 10 sec and 45 subsequent amplification cycles of denaturation at 95°C for 5 sec and annealing/elongation at the temperature indicated in Table 1 for 20 sec. The primer sequences are listed in Table 1. Relative gene expression was calculated by the $2^{-\Delta\Delta C_T}$ method.[28]

RESULTS

Gene expression profile of mammary carcinomas

Three spontaneous and four radiation-induced mammary adenocarcinomas, as well as three normal mammary tissues, were firstly analyzed by expression microarrays carrying 31,024 probe sets, each of which corresponds to one gene sequence. We selected data from 6,926 probe sets for cluster analysis excluding those with an intensity value less than 1,000. Unsupervised hierarchical clustering separated a cluster of spontaneous carcinomas from radiation-induced cancers, whereas the expression profiles of radiation-induced cancers did not form a single cluster (Fig. 1). Changing the threshold intensity value for probe set selection between 50 and 3,000 did not affect the topology of the dendrogram (data not shown), indicating the robustness of the clustering result. Spontaneous and radiation-induced cancers were thus distinguishable based on their global expression profiles.

Genes with differential expression

We then analyzed the intensity data of 6,926 genes to select those that exhibited differential expression between these two types of cancers. First, we searched for genes that had P values < 0.05 by Welch's t-test and simultaneously showed > 2-fold difference in the average intensity between the two groups. We obtained 33 genes that fulfill this criterion, but none of them were increased specifically in radiogenic tumors as compared to normal tissues (Table 2). Because the global gene expression of radiogenic carcinomas were heterogeneous (Fig. 1A), we speculated that radiogenic cancer-specific gene expression would be confined to a subset of radiogenic carcinomas and we might have overlooked such genes using the cutoff value of $P <$ 0.05. We therefore searched for genes that had P values ≥ 0.05 but exhibited > 4-fold higher expression in radiogenic carcinomas as compared to spontaneous ones, identifying 18 additional genes that fulfill this criterion (Table 3).

Clustering analysis (Fig. 1) indicates that most genes were commonly altered in all of seven carcinomas. We examined

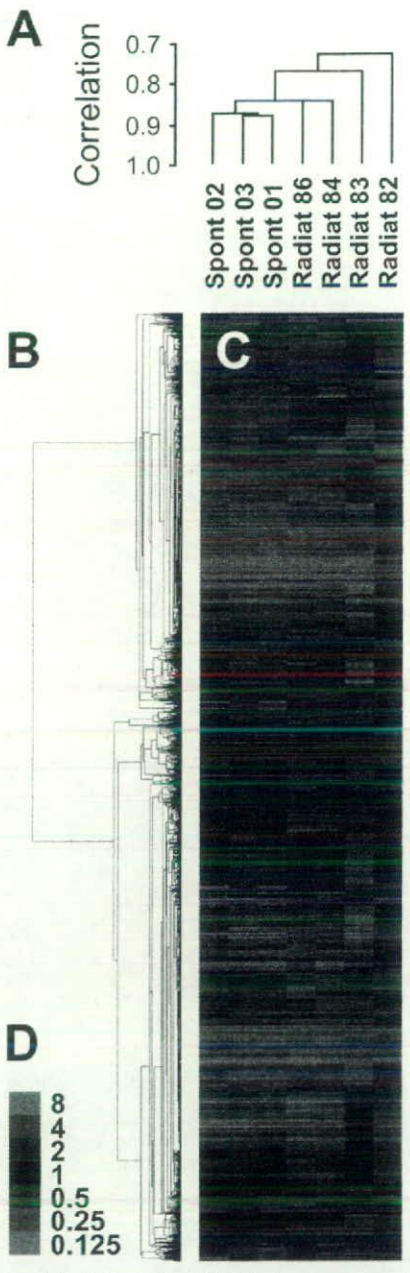

Fig. 1. Hierarchical clustering of global gene expression profiles for rat mammary carcinomas. *A*, Clustering of three spontaneous (*Spont*) and four ionizing radiation–induced (*Radiat*) carcinomas, showing the degree of similarity between tumors. The Pearson correlation coefficient is indicated on the left. *B*, Clustering of 6,926 genes. *C*, Overall expression profiles of 6,926 genes across the seven rat mammary carcinomas. *D*, Color scale for panel *C*.

the average intensity values of seven carcinomas and three normal glands, in which Welch's t-test identified 2,407 genes from the 6,926 probe-set data ($P < 0.05$). We listed the top 30 genes, including 15 of those most increased and 15