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

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 $\beta$ (TGF-$\beta$)-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-$\beta$, 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 in 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 immunohistochemistry 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 (mousy 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% H2O2/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% H2O2 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).

Immunohistochemical 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)-labeled 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 normal 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 minimal 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

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

<table>
<thead>
<tr>
<th>Genes</th>
<th>Directions</th>
<th>Sequences</th>
<th>Gene Bank accession numbers</th>
</tr>
</thead>
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<tr>
<td>Pepsinogen I</td>
<td>Upper</td>
<td>CAACCAACACCGCTTCAACCTGAGGA</td>
<td>BC29055</td>
</tr>
<tr>
<td></td>
<td>Lower</td>
<td>GCGGTCGCGTGGTCTGGAGACTGT</td>
<td></td>
</tr>
<tr>
<td>RUNX3</td>
<td>Upper</td>
<td>GCCAACCCGTCCCCCTACCACCTTACT</td>
<td>NM_004350</td>
</tr>
<tr>
<td></td>
<td>Lower</td>
<td>GCTGCCGGCCACCATGGGAGACT</td>
<td></td>
</tr>
<tr>
<td>ARP</td>
<td>Upper</td>
<td>CAGACCCAGCAGCAGCAGACAGCACCAACCATG</td>
<td>M7885</td>
</tr>
<tr>
<td></td>
<td>Lower</td>
<td>GCTGCCATTGCAACACCTGCTGGATG</td>
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</tbody>
</table>
RUNX3 in chief cell type gastric cancers

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.

<table>
<thead>
<tr>
<th></th>
<th>RUNX3*</th>
<th>Total</th>
<th>P values</th>
</tr>
</thead>
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<tr>
<td></td>
<td>+</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Differentiated adenocarcinomas</td>
<td>24 (45%)</td>
<td>29 (55%)</td>
<td>53</td>
</tr>
<tr>
<td>Undifferentiated adenocarcinomas</td>
<td>21 (43%)</td>
<td>28 (57%)</td>
<td>49</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>45</td>
<td>57</td>
<td>102</td>
</tr>
</tbody>
</table>

*P=0.844

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

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

**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-β, 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.

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; Yamashita 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

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

Acknowledgements. The authors thank Dr. Malcolm A. Moore for revision of the scientific English language. This study was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare, Japan, and a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Accepted July 4, 2008